THE UNIVERSITY OF ALBERTA

Localization and Characterization of a Cell-Associated Precursor to Exocellular Protease 1 of *Pseudomonas aeruginosa*

by

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Supervisor

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Date: November 20, 1981

To my parents, to Sonya and Nicky,

to Tigger,

and to the Montreal Expos

Abstract

Pseudomonas aeruginosa 34362A produces two extracellular proteases, a major protease (protease 1) and a minor protease (protease 2). Although active protease 1 is exclusively found in the culture milieu, an inactive but immunologically cross-reactive precursor to protease 1 has been found to be cell-associated. The nature of this cell association was investigated by cell fractionation studies and immunoelectron microscopy. It was found that the majority of the precursor resides in the periplasmic space, although its association with the cell appears to be more tenacious than the classical periplasmic protein alkaline phosphatase.

Several attempts were made to purify the precursor in its inactive state using ion exchange chromatography and gel filtration, but each purification procedure resulted in the recovery of the precursor as an active protease. Therefore, conditions that would stabilize the precursor (*i.e.*, prevent activation) were investigated. It was found that high ionic strength or low pH buffers stabilized the precursor during dialysis, but when these conditions were used for chromatography spontaneous activation of the precursor still resulted.

It was shown that the precursor in crude cell extract could be activated by several means in addition to

purification. A variety of proteolytic enzymes were found to bring about activation of the precursor to active protease 1, and treatment with the anionic detergents sodium dodecyl sulfate, N-lauroyl sarcosine and deoxycholate also brought about activation of the precursor. Kinetic studies of activation of the precursor by protease 2 and N-lauroyl sarcosine showed that activation was at least a two-step process.

The precursor was purified under denaturing conditions using immunoabsorbance chromatography in order to carry out N-terminus and molecular weight analyses. These findings show that an N-terminal signal region is not required for the secretion of protease 1 through the outer membrane. It was found that the precursor, protease 2-activated precursor and protease 1 were identical with respect to these two parameters. All three proteins were found to have the same molecular weight (33,000) and to contain N-terminal alanine.

The major difference between unactivated precursor and its active counterpart (protease 1) was found to be their surface hydrophobicity, as determined by octyl-Sepharose chromatography. Unactivated precursor was found to be more hydrophobic than the active species.

Studies of protease 1-negative mutants revealed that one such mutant, which did not secrete active protease, did accumulate precursor in its periplasmic space at four times the concentration found in wild-type cells.

It is proposed that the precursor is an intermediate form of protease 1 which is inactive due to its different conformation. This difference in tertiary structure may play an important role in the secretion of the precursor across the outer membrane.

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CHAPTER I

GENERAL INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium which normally has a low virulence for humans. However, some strains have been shown to be involved in various infections in compromised hosts such as burn infections [1,2,3,4] and severe corneal infections [5,6]. It has been shown that strains of *P. aeruginosa* produce several extracellular proteins which are implicated in its pathogenesis; these proteins include a heat-labile exotoxin [7,8], several proteases [9,10], and hemolysins [11].

P. aeruginosa 34362A has been shown to produce two distinct extracellular proteases, protease 1 and protease 2, when grown on TCS broth [12]. Protease 1, the major extracellular protease, is an endoprotease which is active against many soluble proteins such as azocasein, elastin and the non-helical region of native collagen [12]. Much of the work done on nosocomal infections of *P. aeruginosa* has implicated this protease (also known as "elastase") as a virulence factor. Protease 2 is produced in much smaller amounts than protease 1 and has a much lower specific activity against azocasein [12]. Little is known about the physiological role of protease 2. It is interesting to note,

however, that when cells are grown on defined medium, only protease 1 activity can be detected [13]. Protease 1 and protease 2 are not immunologically cross-reactive [12].

Regardless of the medium used to grow *P. aeruginosa* 34362A, *i. e.* either TCS broth or defined medium, no appreciable amounts of protease can be detected in association with the cell at any stage in the growth cycle (S.E. Jensen, personal communication). Of particular relevence to the study to be reported in this thesis is the fact that protease 1 represents one of few clear examples of true extracellular proteins synthesized by Gram-negative bacteria. Despite the fact that much attention has been given to elucidation of the mechanism of protein insertion into and through several membranes (*eg.* endoplasmic reticulum, bacterial cytoplasmic membrane, etc.), little or no attention has been directed toward gaining an understanding of the mechanism of transport of extracellular proteins through the two distinct biological membranes of Gram-negative bacteria. Protease 1 production in *P. aeruginosa* provides a system whereby the latter can be studied in further detail.

Most secreted proteins are water-soluble, yet it is known that most soluble proteins are incapable of spontaneous insertion or passage through membranes. How do

these proteins which have predominantly polar surfaces traverse the hydrophobic center of a lipid bilayer?

The most widely accepted theory for the secretion of proteins across membranes is the "Signal Hypothesis" [14]. Although this hypothesis was derived from studies of eukaryotic cells [14,15,16,17,18], parallels have been observed for the secretion of proteins in prokaryotes. According to the signal hypothesis, initiation of protein synthesis occurs in the cytosol. If the first sequence of amino acid residues emerging from the larger subunit of the ribosome constitutes a "signal region", this leader region will allow for the attachment of the ribosome to the endoplasmic reticulum. The signal region forms a channel through which the growing nascent chain can be inserted into the lumen. It is believed that cleavage of the signal region occurs during polypeptide synthesis, although some exceptions to this have been found. A wide variety of secreted proteins have subsequently been found to be synthesized *in vitro* with a leader sequence of 15-30 hydrophobic amino acids at their N-termini [19,20,21,22].

In the case of bacteria, there is no endoplasmic reticulum, but its functional counterpart for protein secretion is the inner membrane. Polysomes have been shown to be attached to the inner membrane of *Escherichia coli* and

to be rich in mRNA's for periplasmic and outer membrane proteins [23,24]. Several periplasmic and outer membrane proteins have subsequently been studied for *in vitro* synthesis of N-terminal signal regions and several bacterial precursors (pro-proteins) been discovered. Most of the work has been done with *E. coli* and the list of bacterial proteins with known signal peptides includes periplasmic proteins such as arabinose-binding protein [25], maltose-binding protein [26] and alkaline phosphatase [27], and outer membrane proteins such as lipoprotein [28] and ompA protein [29]. There are clear parallels in the roles of eukaryotic and prokaryotic signal peptides: the amino acid sequences are homologous, they are similar in length, in distribution of polar and apolar amino acids, and in the nature of the COOH-terminal residue of the prepiece (for recent reviews, see [30,31]). The structure of the leader sequences is not governed by the final destination of the mature protein, since the sequences are equivalent for proteins to be ultimately located either in the periplasm or in one of the two bacterial membranes. This clearly implies that the N-terminal signal region is required solely for vectorial transport through the inner membrane, and that subsequent location is determined by other factors. The research carried out in this investigation may help to define the nature of these other factors that determine

whether a protein that has traversed the inner membrane should be destined for extracellular secretion, retention in the periplasm or incorporation into the outer membrane. The work to be described may also help to define the mechanism(s) that prevail for the correct insertion of a protein into the outer membrane or expulsion into the extracellular milieu.

A profitable approach for evaluation of the role of signal peptides in bacterial systems has been the use of mutants of *E. coli* that produce hybrid proteins. Probably the most significant finding in this area is the observation that the signal peptide may not be sufficient for the export of proteins through the inner membrane. By means of gene fusion, hybrid proteins were formed containing the N-terminal portion of maltose-binding protein (a periplasmic protein), including its signal sequence, and most of β-galactosidase (a cytoplasmic enzyme) [32]. One would predict that these hybrid proteins would traverse the inner membrane and be primarily located in the periplasm, provided that the N-terminal portion of the molecule dictated its destination. This was not found to be the case; most of the hybrid protein, while able to be partially inserted into the cytoplasmic membrane, were not able to cross the membrane and therefore were largely localized in this membrane [32]. This ultimately would have deliterious effects on the cell

itself as other periplasmic and outer membrane proteins would not be able to be exported and the foreign protein might have other undesirable effects on the function of the inner membrane. Bassford and Beckwith [32,33] have also demonstrated that an additional mutation in the signal sequence of this hybrid protein prevents secretion and, in this case, the hybrid protein is largely located in the cytoplasm.

In the case of β-lactamase (a periplasmic enzyme) secretion in *Salmonella* infected with phage P22, it is known that the protein is synthesized as a precursor molecule [34]. However, mutant precursor proteins lacking as little as 21 amino acids from the C-terminus are unable to be secreted through the cytoplasmic membrane and accumulate in the cytoplasm [34]. Secretion of β-lactamase may therefore depend on the overall conformation of the protein, as described below.

Work done on the insertion of phage M13 coat protein into the inner membrane of *E. coli* has shown that the pro-coat protein is synthesized on free polysomes in a soluble form [35,36]. Insertion into the inner membrane is post-translational, and correct insertion is determined by the overall conformation that the pro-protein assumes as a result of the presence of the hydrophobic prepiece [37,38].

This folding into the membrane forms the basis for the "Membrane Trigger Hypothesis" of Wickner [39]. The crux of this hypothesis lies in the necessity of a protein to be able to assume at least two conformations, one in which its hydrophobic groups are buried in the interior such that it is water soluble, and another in which it has an exposed hydrophobic face that anchors it to the bilayer [39]. This ability of a protein to exist in two stable conformations has been shown to occur with fumarase [40,41], adenylate cyclase [42,43], protein II of *E. coli* outer membrane [44], and *E. coli* pyruvate oxidase [45]. Murphy *et al.* [46] have also found that cholera toxin is synthesized in a precursor form on free ribosomes in *Vibrio cholera* and is secreted post-translationally.

Examples of assembly of proteins that do not seem to depend on the signal peptide give strong support to the theory that other factors may be involved. Those most relevent to this study are reviewed below.

Halegoua and Inouye [47] were able to selectively inhibit the biosynthesis and assembly of three outer membrane proteins of *E. coli* (matrix protein, tolG protein and lipoprotein) by exposing cells to a variety of phenethyl alcohol concentrations. Each of these proteins have been shown to be synthesized as a pro-protein [48,49]. At

phenethyl alcohol concentrations of 0.3% or higher, the processing and assembly of matrix proteins was inhibited, resulting in an accumulation of pro-matrix protein. Pro-matrix protein was able to be secreted through the cytoplasmic membrane but proper insertion into the outer membrane was not achieved. Instead, the pro-matrix protein was found to be only loosely associated with the outer membrane fraction. More importantly, it was shown that at lower phenethyl alcohol concentrations, each of the above-mentioned proteins were properly processed, but the processed proteins (tolG protein and lipoprotein) were accumulated in the periplasmic space. The processed lipoprotein of the soluble fraction (periplasm) was shown to be trypsin-sensitive, in contrast to mature lipoprotein which is trypsin resistant. These results indicate that the precursor protein with the peptide extension is transformed into a new assembly intermediate after the extended peptide is removed. These data indicate that the peptide extension is not essential for the insertion of these outer membrane proteins into this membrane.

Palmiter *et al.* [50,51] have shown that ovalbumin, the main secretory product of oviduct gland cells, does not contain an N-terminal signal sequence. Instead, it appears that ovalbumin contains an internal signal sequence which is recovered in the tryptic fragment comprising residues

229-276 of the mature protein [52]. This tryptic fragment contains a region of sequence homology to the signal sequences of two other oviduct products, ovomucoid and lysozyme. The tryptic fragment has been shown to inhibit translocation of pre-prolactin into dog pancreas membranes [52,53]. This type of internal sequence may be necessary for the post-translational import of proteins from the cytosol into organelles.

Selective uptake of proteins into organelles and their distribution to different compartments has been further investigated in light of the possible involvement of the signal sequence. It is generally believed that protein import into chloroplasts and mitochondria is a post-translational process, not coupled to protein synthesis. It has been shown that many mitochondrial proteins (cytochrome *c* oxidase [54], carbamoyl phosphate synthetase [55], ornithine transcarbamoylase [56] and aspartate amino-transferase [57]) are derived from larger precursors made in the cytoplasm. However, some exceptions to this have been found and are discussed below.

Cytochrome *c* has been shown to be synthesized on free ribosomes, but no precursor for this protein exists [58,59,60]. Cytochrome *c* must traverse the outer mitochondrial membrane to reach its destination at the

cytoplasmic side of the inner membrane. The mechanism of this process is still enigmatic.

Other mitochondrial proteins have been shown to lack precursor forms. These include the ADP/ATP carrier protein of *Neurospora crassa* [61], one of the subunits of cytochrome oxidase [62], and carbamoyl phosphate synthetase from tadpole liver [63]. The mechanism(s) of insertion of these proteins is still under investigation.

P. aeruginosa 34362 has been shown to contain a cell-associated, enzymatically inactive precursor to extracellular protease 1 when grown on TCS broth [64]. This thesis deals with the localization and characterization of the precursor, in order to determine the mechanism of secretion of protease 1.

CHAPTER II

MATERIALS AND METHODS

I. ORGANISMS USED

A. *Pseudomonas aeruginosa* 34362A: This strain of *P. aeruginosa* is pyocine type 5 and was a clinical isolate from a cystic fibrosis patient suffering from pseudomonal pneumnonia. The strain was a gift from Dr. C.H. Pai, Montreal Children's Hospital.

B. *Pseudomonas aeruginosa* PAKS 1 and PAKS 18: These strains were generously provided by Dr. B. Wretlind, Karolinska Hospital, Stockholm, Sweden.

II. GROWTH CONDITIONS

A. Media: Trypticase-Soy Broth (TCS Broth) (Baltimore Biological Laboratories) was used for routine culture and maintenance. Low-phosphate medium containing 0.5% neopeptone (Difco), 0.25% yeast extract (Difco), 0.1% glucose, and 1 m*M* $CaCl_2$, pH 7.2, was used when both alkaline phosphatase and precursor production was required.

B. Incubation Conditions: Routinely, 400 ml amounts of TCS broth or low-phosphate medium in 2 *l* flasks were inoculated to 1% with a 24 hour subculture of the desired

organism. The inoculated medium was incubated for 12 hours (for precursor) or 15 hours (for precursor and alkaline phosphatase) at 30°C on a gyratory shaker (300 rpm).

Stock cultures were stored lyophilized or maintained on TCS broth + 1.5% agar plates with monthly transfers.

III. ASSAYS

A. Protease: The reaction mixture consisted of 1 ml 0.02 *M* Tris-HCl, 2 m*M* $CaCl_2$, pH 7.5, and enzyme sample (0-50 *u*l). The reaction mixture was prewarmed to 37°C, and to this mixture was added 1 ml of prewarmed 1% azocasein, pH 7.5. The reaction was allowed to proceed for 15 min, after which the reaction was stopped by the addition of 2 ml of cold 10% trichloroacetic acid. After 30 min, the stopped reaction mixture was filtered through a Whatman No. 1 paper followed by measurement of the A_{370} of the supernatant. One unit of protease is defined as that amount which would give an increase in absorbance at 370 nm of 1.0 in 1 min under these standard assay conditions.

B. Precursor : The reaction mixture consisted of 1 ml of 0.02 *M* Tris-HCl, 2 m*M* $CaCl_2$, pH 7.5, precursor sample (0-50 *u*l) and 10 *u*l of protease 2 (2 mg/ml). The reaction mixture was incubated at 37°C for 15 min after which the mixture was assayed for new proteolytic activity by the

addition of azocasein, as described above. One unit of precursor is defined as that amount which upon limited proteolysis gives rise to one unit of new protease activity, as defined above.

C. Alkaline Phosphatase: This assay procedure was carried out according to the method of Neu and Heppel [65]. The 1 ml reaction mixture consisted of 0.05 ml of enzyme plus H_2O, 0.9 ml 1 *M* Tris-HCl, pH 8.0, and 0.05 ml of 20 m*M* *p*-nitro-phenylphosphate. The reaction was started with the addition of substrate and allowed to proceed for 5 min, after which the reaction was stopped by the addition of 4 ml of 0.01 *M* potassium phosphate, pH 10 and the A_{410} was read.

D. Glucose-6-phosphate Dehydrogenase: This assay was carried out according to the procedure of Malamy and Horecker [66]. The reaction mixture consisted of 1 ml containing 0.05 *M* Tris-HCl, pH 7.65, 0.01 *M* $MgCl_2$, 1 *u*mole glucose-6-phosphate, 0.4 *u*mole NADP, and the reaction was started by the addition of enzyme. NADP reduction to NADPH (at 25°C) was followed at 340 nm.

E. Protein Determination: Protein concentration was routinely determined by i. the procedure of Lowry *et al.* [67] using bovine serum albumin as the protein standard or ii. by measuring A_{280}.

IV. CELL FRACTIONATION PROCEDURES

A. Mechanical Breakage in French Pressure Cell: *P. aeruginosa* 34362 A, grown in TCS broth, were harvested after 12 hours of growth (for maximum precursor activity), were then washed two times with 0.01 *M* Tris-HCl, pH 7.5, and cells were resuspended in the same buffer to 1/50 the original culture volume. The cells were then passed through a French pressure cell (15,000 psi) once. The resulting cell homogenate was then centrifuged at 105,000 *g* for 1 hr. The supernatant fluid is referred to as the "crude cell extract", and the pellet is the "cell wall fraction".

The same procedure was used in the localization studies, excepts cells were grown on low phosphate medium and were harvested after 15 hours of growth.

B. Grinding with Plastic Beads: *P. aeruginosa* cells were grown, harvested and washed as described above. Cells were resuspended in 25 ml of 0.01 *M* Tris-HCl, pH 7.5, and to this suspension was added 25 g of plastic beads (SM-Biobeads, 20-40 mesh). Cells were subjected to grinding in an Omnimixer at maximum speed for 20 min. The cell-plastic bead mixture was filtered through glass wool and then centrifuged for 15 min at 27,000 *g*. The supernatant was then centrifuged for 1 hour at 105,000 *g*. The resulting supernatant was then assayed for precursor activity.

C. Sonic Oscillation: *P. aeruginosa* cells were grown, harvested and washed as above. Resuspended cells (approx. 10 ml) were treated three times for 30 sec intervals in a Bronwell Brosonik III sonic oscillator with a 100 energy tip. Cells were then centrifuged at 105,000 *g* for 1 hour and the supernatant was assayed for precursor activity.

D. Spheroplast Formation: Spheroplasts were formed according to the procedure of Cheng *et al*. [68]. *P. aeruginosa* cells were grown in 80 ml low-phosphate medium and were harvested after 15 hours of growth. Cells were washed two times in 0.01 *M* Tris-HCl, 0.01 *M* $MgCl_2$, pH 7.5, and were resuspended in 40 ml of 0.01 *M* Tris-HCl, 0.2 *M* $MgCl_2$, 0.5 mg/ml lysozyme, pH 8.4. The mixture was then incubated on a shaker at room temperature for 30 min, followed by centrifugation at 12,000 *g* for 10 min. The resulting supernatant is referred to as "spheroplast extract". The cells were examined with the phase contrast microscope to confirm the presence of spheroplasts. The cells were then resuspended in 10 ml of 0.01 *M* Tris-HCl, 0.01 *M* $MgCl_2$, pH 8.4, centrifuged again for 10 min at 12,000 *g* and the resulting supernatant is referred to as "spheroplast shock fluid". The cells were then used to prepare "spheroplast cell extract" and "spheroplast cell envelopes" by mechanical breakage in the French pressure cell as described above.

E. Osmotic Shock: Periplasmic proteins were defined as those released by the high $MgCl_2$ shock procedure described by Cheng *et al.* [69]. Cells grown in 80 ml Low-phosphate medium were washed and resuspended in 10 ml of 0.01 *M* Tris-HCl, 0.2 *M* $MgCl_2$, pH 8.4. The mixture was incubated at 37°C for 15 min with frequent shaking and centrifuged at 12,000 *g* for 10 min (supernatant = "$MgCl_2$ extract"). The pellet was resuspended in 0.01 *M* Tris-HCl, 0.01 *M* $MgCl_2$, pH 8.4, incubated at 37°C for 10 min and centrifuged at 12,000 *g* for 10 min (supernatant = "$MgCl_2$ shock fluid"). The resulting shocked cells were used to prepare "shocked cell extract" and "shocked envelopes" by mechanical breakage in the French pressure cell as described above.

F. Preparation of Purified Protease 1: Cells were harvested after 16 hours of growth and the supernatant collected ("culture supernatant"). The supernatant, containing over 99% of the total protease 1 activity, was fractionated and the protease 1 was purified as previously described [12].

G. Preparation of Purified Protease 2: Protease 2 was isolated and purified as previously described [12].

H. Protease 2-Activated Precursor: Purified protease 2 was added to a preparation of unactivated precursor (cell extract) in the proportion of 11 *u*g protease 2 per unit of

precursor and was incubated at 37°C until all of the precursor had been converted to active protease (approximately 15 min).

I. Preparation of Deoxycholate-Activated Precursor: Equal volumes of crude unactivated precursor and 1% (w/v) deoxycholate were mixed and incubated at 37°C for 15 min, by which time in excess of 95% of the precursor had been converted to active protease.

V. PROTEIN SEPARATION TECHNIQUES

A. Ammonium Sulfate Fractionation: Saturated $(NH_4)_2SO_4$ solution was added with continuous mixing to a sample of crude cell extract to yield 35% saturation. The pH was maintained at 7.5. After stirring for 30 min at 4°C, the mixture was centrifuged for 15 min at 27,000 *g*, the supernatant fluid collected,and saturated ammonium sulfate solution was added to yield 55% saturation. The solution was stirred as before. The resulting precipitate was collected by centrifugation and dissolved in a suitable amount of 0.01 *M* Tris-HCl, pH 7.5. One ml samples of the dissolved precipitate were dialyzed against 2 *l* of each of the buffers shown in Table VI at 4°C for 16 hours. Samples were then assayed for precursor and protease.

B. DEAE-Cellulose Chromatography: Ten ml of crude cell extract (37 units of precursor) were applied to a column (1.6 x 35 cm) of DEAE-cellulose that had been previously equilibrated with 0.01 *M* Tris-HCl, pH 7.5. The column was first washed with 100 ml of the above buffer, then eluted with a 300 ml linear gradient of 0 to 0.5 *M* NaCl in 0.01 *M* Tris-HCl, pH 7.5. Fractions (5 ml) were collected, and those containing activated precursor were pooled and applied to a second DEAE-cellulose column (1.6 x 20 cm), which was developed in the same fashion.

C. CM-Cellulose Chromatography: A 3 ml sample of an $(NH_4)_2SO_4$ fraction of crude precursor was dialyzed against 2 *l* of 0.01 *M* sodium acetate, pH 5.6 and 2.5 ml (7.2 units of precursor) were applied to a CM-cellulose column (1.6 x 35 cm), pre-equilibrated in the same buffer. The column was first washed with 150 ml of the above buffer, followed by elution with a 300 ml linear gradient of 0 to 0.5 *M* NaCl in 0.01 *M* sodium acetate, pH 5.6. Five ml fractions were collected and were assayed for precursor and protease activity.

D. Sephadex G-100 Gel Filtration: Five ml of crude cell extract containing 14 units of precursor was applied to a 1.6 x 40 cm Sephadex G-100 column. Protein was eluted with 0.01 *M* Tris-HCl, pH 7.5 and 5 ml fractions were collected

and assayed for precursor and protease activity.

E. Sephadex G-100 Gel Filtration (in $MgCl_2$): A 3 ml $(NH_4)_2SO_4$ fraction of crude precursor was dialyzed overnight against 2 l of 0.01 *M* Tris-HCl, 0.5 *M* $MgCl_2$, pH 7.5. 2 ml (8.6 units of precursor) of the dialyzed sample was applied to a 1.6 x 40 cm Sephadex G-100 column which had been pre-equilibrated in the same buffer. 5 ml fractions were collected and were assayed for precursor and protease activity.

F. Octyl-Sepharose Chromatography: A 35-55% $(NH_4)_2SO_4$ sulfate insoluble fraction of crude cell extract containing 54 units of precursor was dissolved to a final volume of 6 ml in 25% $(NH_4)_2SO_4$, 0.01 *M* Tris-HCl, pH 7.5. 3 ml were treated with 280 *u*g of protease 2, resulting in 100% activation of precursor, and the remaining 3 ml were left untreated. Both the treated and untreated preparations were applied to separate but similar octyl-Sepharose columns (1.2 x 25 cm) that had been pre-equilibrated in the above buffer. The columns were washed with 100 ml of the pre-equilibration buffer, and gradient elution was begun with fraction 21. The linear gradient consisted of an increasing concentration of ethylene glycol (0-50%) and a decreasing concentration of $(NH_4)_2SO_4$ (25-0%) in 0.01 *M* Tris-HCl, pH 7.5. Five ml (80 drop) fractions were collected and assayed for precursor and

protease activity, and for $(NH_4)_2SO_4$ concentration using Nessler's reagent.

G. Glycerol Density Gradients: Six 5-25% (v/v) linear gradients of glycerol in 0.01 *M* Tris-HCl, pH 7.5 were prepared with a conical gradient maker (final vol. = 13.3 ml). Cell extract (200 *u*l) containing precursor were layered onto three of these gradients, and 200 *u*l of pure protease 1 were layered onto the other three. The gradients were then centrifuged in a SW40.1 rotor at 40,000 rpm for 24 hours at 4°C in a Model L ultracentrifuge (Beckman Instruments). Fractions (25 drops/fraction) were collected from the bottom of the tubes using a 21 gauge needle and were immediately chilled on ice. Two adjacent gradients (*i.e.*, one containing cell extract and one containing protease 1) were then assayed for precursor and/or protease 1 activity. This procedure was repeated three separate times. In addition, the same procedure was carried out using cell extract activated by either protease 2 or deoxycholate instead of untreated cell extract.

VI. PURIFICATION OF IgG FROM WHOLE RABBIT SERUM

A. Preparation of Immune Serum: New Zealand white rabbits were immunized with protease 1 as described by Jensen *et al.* [12].

B. Ammonium Sulfate Fractionation: Saturated $(NH_4)_2SO_4$ was added to 25 ml of whole serum (anti-protease 1 antiserum or normal serum) to give a final concentration of 40% saturated $(NH_4)_2SO_4$ (in 0.01*M* potassium phosphate, pH 8.0). This solution was mixed at 4°C for 30 min and then was centrifuged at 27,000 *g* for 15 min. The pellet was dissolved in 25 ml of cold distilled water and saturated $(NH_4)_2SO_4$ was added to a final concentration of 40% saturation. This entire procedure was repeated four times. The final pellet was resuspended in 10 ml of 0.01 *M* potassium phosphate, pH 8.0, and then the sample was dialzyed twice against 2 l of the above buffer.

C. DEAE-Cellulose Chromatography: 5 ml of the dialyzed sample was added to a 1.6 x 30 cm DEAE-cellulose column which had been pre-equilibrated with 0.01 *M* potassium phosphate, pH 8.0. The column was washed with 250 ml of the above buffer to remove IgG which did not bind to the column. The remaining bound protein was eluted with 1 *M* NaCl in 0.01 *M* potassium phosphate, pH 8.0. The IgG peak tubes were pooled and concentrated to a final volume of 5 ml using an Amicon UM-50 filter.

VII. OTHER IMMUNOLOGICAL PROCEDURES

Immunoglobulins, partially purified by $(NH_4)_2SO_4$ fractionation (see above), were used for the following:

A. Immunoprecipitation of Protease 1, Inactive Precursor, and Protease 2-activated Precursor: A 35-55% $(NH_4)_2SO_4$ fraction of untreated cell extract (inactive precursor) and of protease 2-activated cell extract (activated precursor) was first prepared. Culture supernatant was used for immunoprecipitation of protease 1. The partially-purified anti-protease 1 immunoglobulins were added to the above samples in the ratio of 1 ml antibody to 25 units of precursor or protease 1, and the mixtures were incubated at 4°C for 16 hr. The precipitates thus formed were collected by centrifugation and washed twice with 0.05 *M* Tris-HCl, 0.85% NaCl, pH 7.5. The final pellets were solubilized at 100°C for 5 min in 200 µl of 2% SDS and 10% glycerol in 62.5 m*M* Tris-HCl, pH 6.8 and subjected to electrophoresis.

B. Preparation of Cyanogen Bromide-linked Sepharose-Anti-protease 1 for Immunoabsorbance Chromatography: Sepharose 4B was activated with cyanogen bromide (40 mg/ml Sepharose) using the method of Mannik and Stage [70]. This was coupled to partially purified anti-protease 1 using the ratio of 2 mg of protein to 1 ml of activated Sepharose.

C. Immunoabsorbance Chromatography: A 1.5x25 cm column was packed at 4°C in 0.05 *M* Tris-HCl, pH 7.5. The capacity of the column was determined by applying excess pure protease 1 and calculating the number of units that bound to the column. This was found to be about 70 units of protease 1, corresponding to approximately 3 mg of protein. Thereafter, comparable units of unactivated precursor from cell contents, protease 2-activated precursor from cell extract and protease 1 from culture supernatant were added to the same column for purification. Non-adsorbed protein was removed by washing with 0.05 *M* Tris-HCl, pH 7.5,and elution of protein was monitored by measuring A_{280}. When no more protein was eluted, the column was washed with 5% formic acid until no further A_{280} absorbing material was eluted. The adsorbed proteins were then removed by eluting with 0.03 *N* NaOH. 2.5 ml fractions were collected in tubes containing 2.5 ml of 10% formic acid. The protein peaks were located and the peak corresponding to the desired protein (as identified by gel electrophoresic mobility) was pooled and dialyzed extensively against 5% formic acid. Protein content of the pooled fractions was estimated by A_{280}. Volumes containing approximately 50 μg of protein were freeze-dried and the protein was solubilized at 100°C for 5 min in 100 μl of 2% SDS, 10% glycerol and 1% β-mercaptoethanol in 0.125 *M* Tris-HCl, pH 6.8 and

subjected to electrophoresis.

VIII. IMMUNOELECTRON MICROSCOPY

A. Preparation of Ferritin-IgG Conjugate: This was carried out according to the method of MacAlister *et al.* [71,72]. Commercial ferritin (6x crystallized) was purchased from Miles Laboratories. Approximately 5 mg of purified IgG was dialyzed against 1 l of 0.1 *M* sodium phosphate, pH 6.8. Three times as much ferritin was centrifuged at 100,000 *g* for 2 hours and the resulting pellet was resuspended in the dialyzed IgG. To this was added a 1/10th volume of 0.4% glutaraldehyde and the mixture was allowed to react for 45 min at room temperature. The coupling reaction was stopped by the addition of 1 drop of 1 *M* $(NH_4)_2CO_3$, pH 8.8. The mixture was held at room temperature for 1 hour to ensure the cessation of the coupling reaction, and then centrifuged at 12,000 *g* for 10 min to remove large aggregates. The supernatant was applied to a Sepharose 6B column (1.6 x 100 cm) that was previously equilibrated with 0.05 *M* sodium phosphate, 0.85% NaCl, pH 6.8. Five ml fractions were collected, and their absorbance at 440 nm was measured.

B. Glutaraldehyde Fixation: Approximately 100 ml of either 12 or 24 hours cells were washed twice in 0.01 *M* sodium phosphate, pH 6.8. The final cell pellet was

resuspended in 4 ml of this buffer, and to this was added 1 ml of 1% glutaraldehyde (final concentration = 0.2%) Cells were fixed for 20 hours at 4°C, and then washed three times in the same buffer. The final pellet was allowed to stand overnight at 4°C, and it was then resuspended in 1.1 *M* sucrose, 0.01 *M* sodium phosphate, pH 6.8 two times for 20 and 25 min, respectively. The cells were finally centrifuged at 27,000 *g* for 10 min and a small sample of the cell pellet was placed on the end of a copper chuck. The chuck was the immersed into Freon 22, cooled with liquid nitrogen, and allowed to freeze for 8-10 sec. The chuck was then transferred to the chuck-holder of the sectioning device.

C. Frozen Thin Sections: Sectioning was performed using glass knives on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc.), equipped with a Sorvall-Christensen FTS/LTC-2 Frozen Thin Sectioner. The low temperature controller of the sectioning device was set to maintain a temperature of -70°C. Sections were transferred from the knife edge to formvar-coated grids by means of a droplet of 2.3 *M* sucrose on an eyelash. The grid bearing the sections in the sucrose droplet was then floated, section side down, on a drop of distilled water on Parafilm.

D. Application of Conjugate to Frozen Thin Sections: The grids were washed with 0.1 *M* sodium phosphate buffer

(pH 7.4) and picked up with non-capillary forceps. A drop of 5% bovine serum albumin was placed on the section side of each grid, which was then placed inside a beaker containing wet filter paper for hydration for 5-10 min. The excess bovine serum albumin solution was then removed and replaced with a drop of conjugate, and the grids were returned to the beaker for a further 5-10 min incubation. Excess conjugate was then removed and the grids were touched to three droplets of buffer on Parafilm in rapid succession and then washed five times in drops of buffer. The grids were then thoroughly washed with water, dried, and examined with the electron microscope.

IX. ELECTROPHORESIS

Slab gel electrophoresis in the presence of SDS was carried out using 20% acrylamide according to method of Anderson *et al*. [73]. The gels were stained for protein with Coomassie Blue.

X. N-TERMINAL ANALYSES

N-terminal analysis was performed using 200 *u*g each of protease 1, unactivated precursor and protease 2-activated precursor according to the dansyl chloride method of Gray [74], with 200 *u*g of lysozyme (N-terminal lysine, Ref. 75) run simultaneously as a control.

CHAPTER III

IDENTIFICATION AND LOCALIZATION OF A CELL-ASSOCIATED PRECURSOR TO PROTEASE 1

I. RESULTS

A. Serological Identification: It has been previously documented that protease 1 activity is exclusively associated with the culture supernatant[64]. Little or no proteolytic activity has been shown to be present in crude cell extract derived from mechanical breakage of washed cells. However, when crude cell extract was tested for immunological cross-reactivity with anti-protease 1 by double diffusion immunoprecipitation, it was found that cell extract obtained from 12 hours cells contained a considerable amount of cross-reacting material, as shown in Plate I. On the other hand, cell extract from 24 hours cells contained much less cross-reacting material. When 12 hours cell extract was assayed using the radioimmunoassy specific for protease 1, the extract was shown to contain appreciable amounts of material which, although enzymatically inactive, was none the less serologically cross-reactive with anti-protease 1[64]. The material that was enzymatically inactive but immunologically related to protease 1 is

PLATE I Ouchterlony double-diffusion plate of antiserum to protease 1.

Well 1 = Pure protease 1

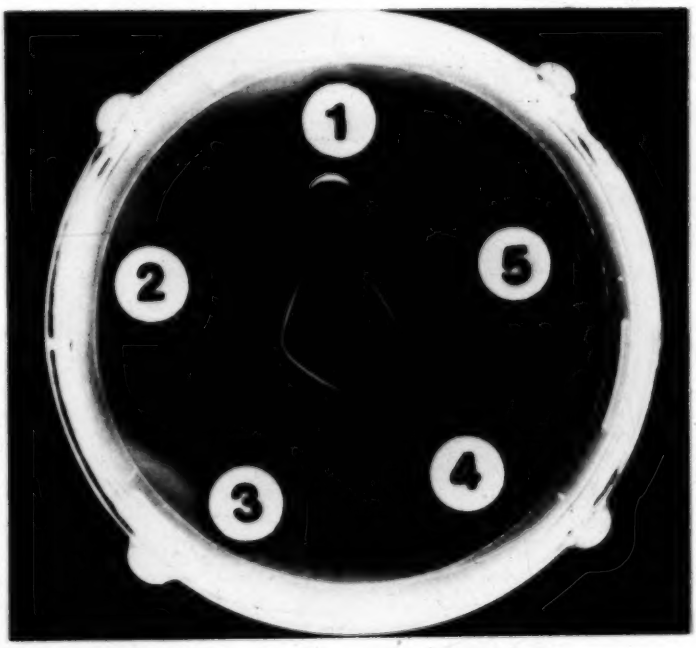
Well 2 = Crude cell extract from 12 hour cells

Well 3 = Culture supernatant from 12 hour cells

Well 4 = Crude cell extract from 24 hour cells

Well 5 = Culture supernatant from 24 hour cells

Centre well = Partially purified anti-protease 1 IgG


1
2
3
4
5

henceforth referred to as "precursor".

B. Proteolytic Activity in Cell Lysate Before and After Addition of Protease 2: The precursor material in cell extract is converted to active protease 1 upon addition of a variety of proteolytic enzymes (see Chapter VI for detailed discussion). Table 1 shows the results of activation of precursor in cell extract by protease 2. In any given cell extract, minimal proteolytic activity is detectable before addition of protease 2. However, after protease 2 is added (20 ug/10 ul cell extract) there is a resulting 20-40 fold increase in proteolytic activity. The proteolytic activity of protease 2 on azocasein is negligible (<10%).

C. Evaluation of Extraction Procedures for Precursor Quantitation:

Washed cells were broken by three methods: mechanical breakage of cells in a French Pressure Cell, disruption of cells by sonic oscillation, and grinding of cells with plastic beads. The resulting cell extracts were assayed for precursor as described in Chapter II. Table II shows that breakage of cells in a French Pressure Cell yielded the greatest recovery of precursor, 3.8 times more than grinding and 1.6 times more than sonic disruption. Precursor in the crude cell extract obtained by mechanical breakage is stable in the inactive state for an extensive period of time (*i.e.*,

Table I

Proteolytic Activity in Cell Extract
Before and After Addition of Protease 2

	Activity (units/ml) (-) Protease 2	Activity[a.] (units/ml) (+) Protease 2	Fold Increase in Activity
Cell Extract 1[b.]	0.3	6.38	21
Cell Extract 2[b.]	0.32	7.96	25
Cell Extract 3[b.]	0.06	2.45	40

a. Sample calculation:
A_{370} of protease 2 alone = 0.04
A_{370} of protease 2 + cell extract = 0.465
$\therefore A_{370}$ of cell extract = 0.425

b. Cell extracts were obtained by mechanical breakage in a French pressure cell as described in Chapter II. The cell extract preparations listed here represent only three of many preparations made and are intended to represent typical preparations.

Table II

Evaluation of Extraction Procedures
for Precursor Quantitation

	Precursor (units/ml)[a.] in Cell Extract
Mechanical Breakage with French Pressure Cell	4.37
Cell Grinding with Plastic Beads	1.13
Sonic Oscillation	2.27

a. Precursor was quantitated after the addition of protease 2 and equivalent cell numbers/ml final volume were disrupted in each case.

greater than 1 year at -20°C).

D. Kinetics of Growth, and Production of Precursor and Protease 1 in TCS Broth: In order to determine the age of cells yielding maximum precursor, a growth experiment was carried out in which samples of *P. aeruginosa* 34362A in TCS broth were taken every three hours over a period of 24 hr. Growth was measured turbidimetrically for each sample and the cells harvested by centrifugation. The culture supernatant was assayed for protease 1 and the cell extract was assayed for precursor. Maximum cell-associated precursor was found to occur at about 12 hr, preceeding maximum protease 1 production which was observed after 24 hours of growth (Figure 1). The decrease in accumulated precursor after 12 hours corresponds to a concomitant increase in extracellular protease 1. Figure 1 represents one of several growth experiments performed. Therefore cells were subsequently harvested after 12 hours of growth on TCS broth to obtain maximum precursor yield.

E. Localization of Precursor: Since active enzyme is >99% extracellular and precursor is entirely cell-associated, it was important to determine the intracellular location of the precursor and the nature of its association with the cell in order to shed light on the mechanism of secretion of precursor. Localization of precursor was

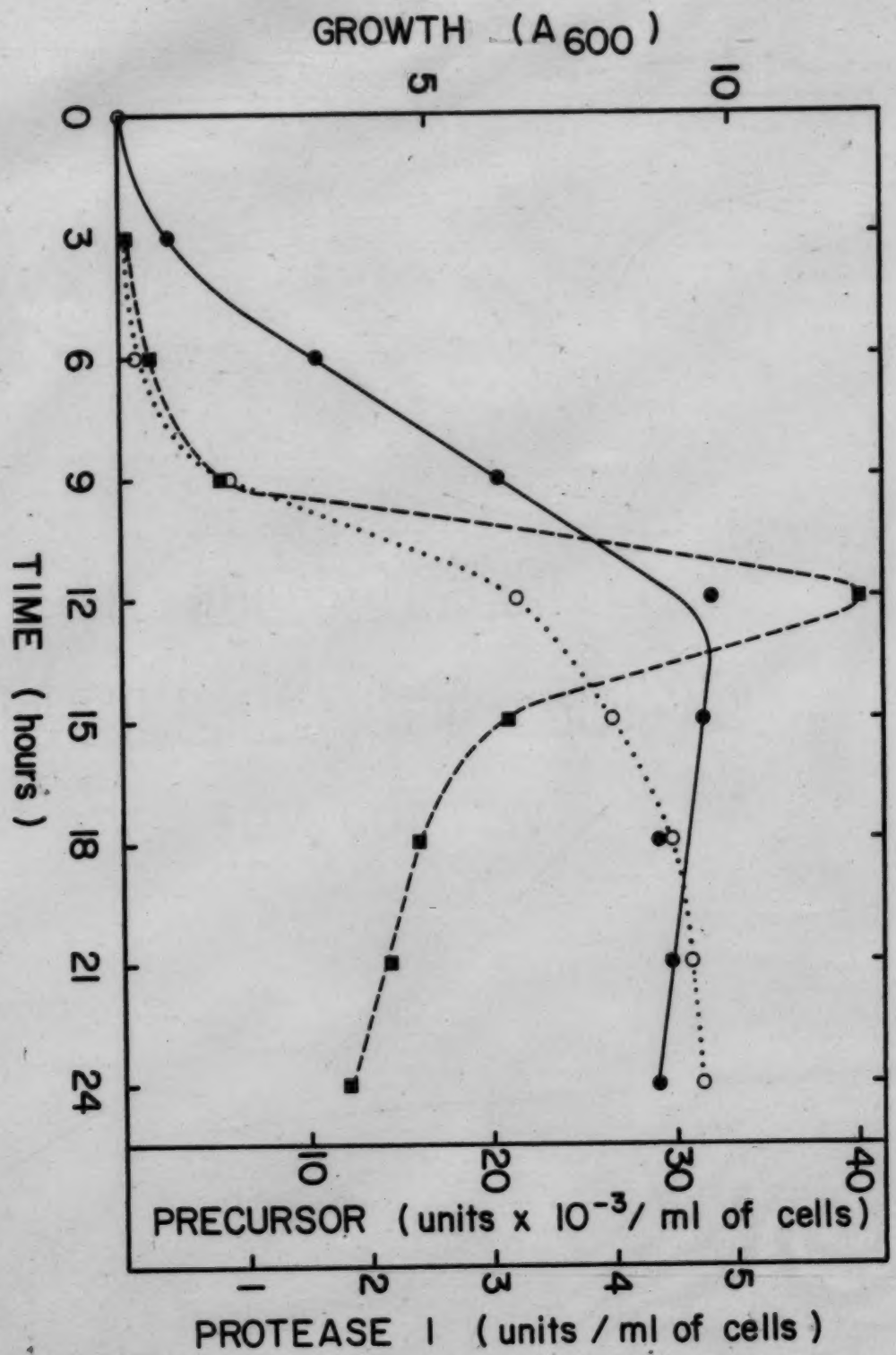
Figure 1. Kinetics of Exocellular Protease and Cell-Associated Precursor Production in a Growing Culture of *P. aeruginosa* 34362A.

Samples were taken at 3 hours intervals from a TCS broth culture. Culture supernatants were assayed for protease activity. Cell extract was prepared from washed cells by mechanical breakage in a French Pressure Cell and assayed for precursor.

Growth ($A_{...}$): ●——●

Precursor (units/mg protein): ■–––■

Protease (units/ml culture supernatant): O····O


GROWTH (A600)
5
10
0
3
6
9
12
15
18
21
24
TIME (hours)
10
20
30
40
PRECURSOR (units x 10-3/ ml of cells)
1
2
3
4
5
PROTEASE I (units / ml of cells)

investigated in the two following ways:

(i) Cell fractionation studies

(ii) Immunoelectron microscopy

(i) Cell Fractionation Studies: The usual method of releasing periplasmic enzymes from *Escherichia coli* cells involves osmotic shock in the presence of EDTA [76] or spheroplast formation by the action of lysozyme and EDTA [66]. Because *P. aeruginosa* is lysed by EDTA [77], implying that EDTA must have a very profound effect on the cytoplasmic membrane, alternative methods for the removal of periplasmic proteins were used. The procedures used to obtain various cell fractions are as follows: mechanical breakage of cells using a French Pressure Cell, spheroplast formation by the action of lysozyme and 0.2 *M* $MgCl_2$, and osmotic shock using 0.2 *M* $MgCl_2$. Details of these procedures are outlined in Chapter II. Cells for this experiment were grown in low phosphate complex medium, and the fractions were assayed for each of the following: precursor, alkaline phosphatase, a known periplasmic protein[68], and glucose-6-phosphate dehydrogenase, a known cytoplasmic protein [66]. The results are summarized in Table III. It can be seen that most of the precursor (84%) but relatively little alkaline phosphatase (7%) was found in the cell extract when cells were broken in a French Pressure Cell. The distribution of precursor was similar to that of glucose-6-phosphate

Table III

Biochemical Localization of Precursor in Whole Cells

Cell Fraction	Precursor[a.] (% of Total)	Alkaline Phosphatase (% of Total)	Glucose-6-P Dehydrogenase (% of Total)
1. Mechanical Breakage in FPC*			
Cell Extract	84	7	85
Cell Envelopes	16	93	15
2. Spheroplasts			
Spheroplast Extract	4	89	1
Spheroplast Shock Fluid	76	5	3
Cell Extract	14	1	81
Cell Envelopes	6	5	15
3. Osmotic Shock			
$MgCl_2$ Extract	8	87	1
$MgCl_2$ Shock Fluid	58	5	2
Cell Extract	24	1	82
Cell Envelopes	10	7	15

a. Precursor was quantitated after addition of protease 2

* French pressure cell

dehydrogenase which at first suggested that precursor might be cytoplasmic rather than periplasmic in location. To verify this, periplasmic proteins were selectively removed without damage to the cytoplasmic membrane. Osmotic shock treatment released 66% of the precursor ($MgCl_2$ extract + $MgCl_2$ shock fluid), compared to only 3% of glucose-6-phosphate dehydrogenase. Furthermore, when spheroplasts were formed (Plate II) 80% of the precursor was released from cells (spheroplast extract + spheroplast shock fluid) while no appreciable release of glucose-6-phosphate dehydrogenase was detected. These results indicate that precursor is not a cytoplasmic component.

Alkaline phosphatase is readily released (87%) by osmotic shock. Precursor, however, requires secondary shock (exposure to low concentration of $MgCl_2$) before its release occurs, and even after this treatment some of the precursor (34%) remains cell-associated. More precursor is released upon spheroplast formation than by osmotic shock (only 20% remains cell-associated) but still the release of precursor differs from the release of alkaline phosphatase. These observations suggest that precursor is envelope-associated but that its association is more tenacious than that of alkaline phosphatase.

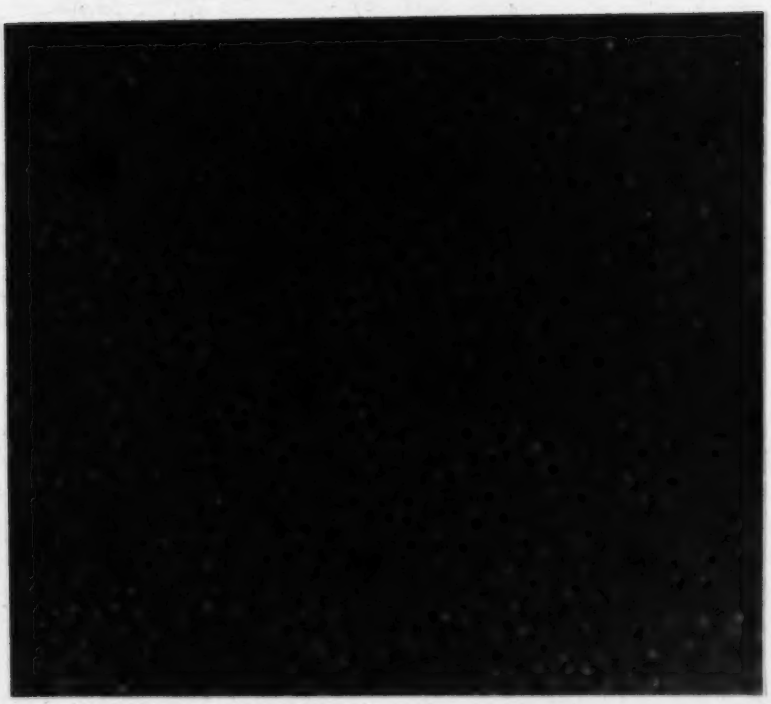
PLATE II Spheroplast formation of *P. aeruginosa* 34362A in 0.2 *M* $MgCl_2$.

A: 15 hour *P. aeruginosa* cells, washed twice with buffer and viewed under the phase contrast microscope.

B: 15 hour *P. aeruginosa* cells, treated with 0.1 mg/ml lysozyme as described in Chapter II. Cells were viewed under the phase contrast microscope.

A

B



It should be noted that precursor released from cells by osmotic shock is much less stable than precursor in crude cell extract, and that it becomes converted spontaneously to active protease within a few days at -20°C.

(ii) Immunoelectron Microscopy:

(a) Preparation of Anti-Protease 1 IgG and Normal IgG: Anti-protease 1 antiserum was obtained from immunized rabbits. Normal rabbit serum, lacking anti-protease 1 antibody, was used as a control. Purification of IgG from both sera was carried out as described in Chapter II. Figures 2 and 3 show the elution profiles from DEAE-cellulose of anti-protease 1 IgG and normal IgG, respectively. The protein that is not retained on DEAE-cellulose (Peak I) is pure IgG. To test for the presence of anti-protease 1 IgG, a ring precipitin test was performed using pure protease 1 as the antigen and peak I of Figure 2 as the antibody. A positive ring precipitin test resulted, confirming that Peak I is anti-protease 1 IgG. Similarly, the presence of normal rabbit IgG was confirmed by using Peak I from Figure 3 as the antigen, and reacting it with goat anti-rabbit IgG antiserum (see Fig.3). Normal rabbit IgG gave no precipitin band when tested with protease 1.

Figure 2. DEAE-Cellulose Elution Profile of Partially Purified Anti-protease 1 IgG.

5 ml of an $(NH_4)_2SO_4$ fraction of anti-protease 1 antiserum were applied to a DEAE-cellulose column (see Chapter II). Peak I represents purified IgG and Peak II other contaminating proteins.
Inset: Positive ring precipitin test to establish the presence of anti-protease 1 IgG.

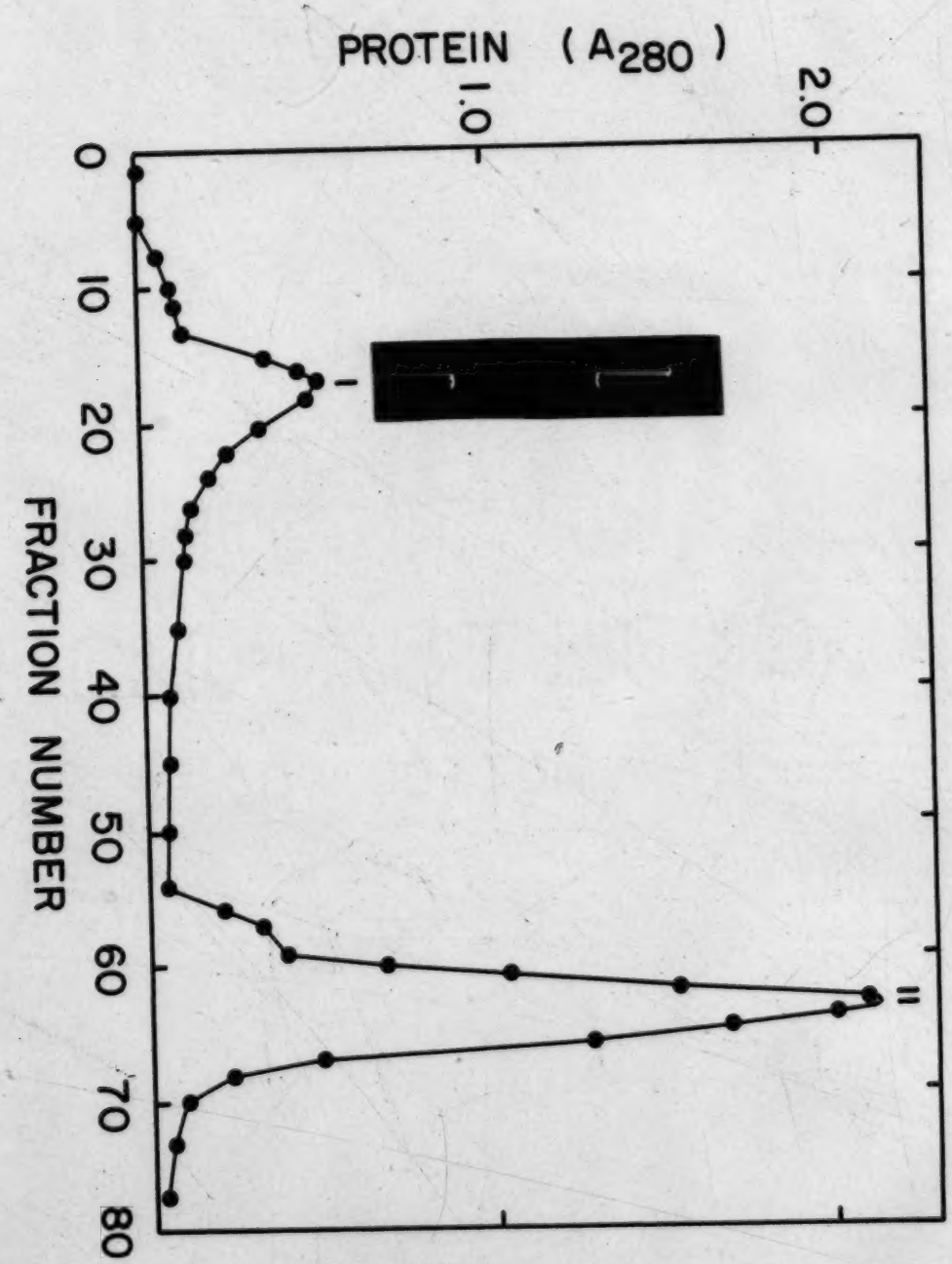
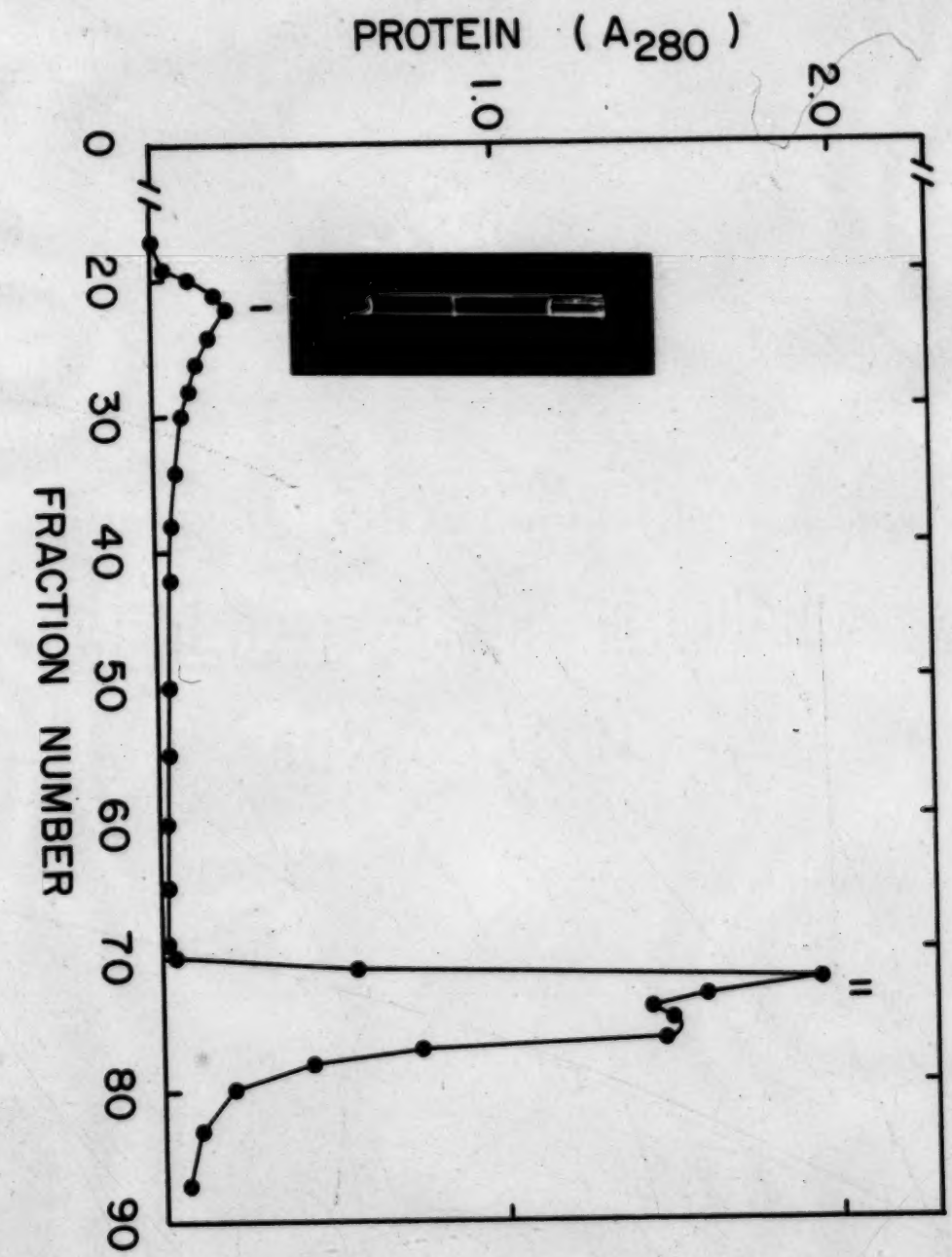

PROTEIN (A280)
1.0
2.0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER
I
II

Figure 3. DEAE-Cellulose Elution Profile of Partially Purified Normal Rabbit Serum IgG.

5 ml of an $(NH_4)_2SO_4$ fraction of normal rabbit serum were applied to a DEAE-cellulose column (see Chapter II). Peak I represents purified IgG and Peak II other contaminating proteins. Inset: Positive ring precipitin test to establish the presence of IgG.


PROTEIN (A280)
1.0
2.0
0
20
30
40
50
60
70
80
90
FRACTION NUMBER
I
II

(b) Conjugation of Purified IgG with Ferritin: The procedure used for preparation of IgG-ferritin conjugate is outlined in Chapter II. Elution of the conjugate from a Sepharose 4B column produced two discernible peaks at 440 nm (Figure 4), a fast moving, high molecular weight peak (Peak I) and a slower-moving peak (Peak II) which exhibited a shoulder at the leading edge. Fractions were collected from the shoulder to be used for reaction with frozen thin sections.

(c) Preparation of *P. aeruginosa* Cells for Thin Sectioning: In order to validate any results obtained from localization of precursor by this technique, it was necessary to include a negative precursor control. Since cells grown in TCS broth at 37°C for 24 hours no longer contained detectable precursor and the extract from these cells gave no precipitin line when reacted with anti-protease 1 IgG (Plate III), these 24 hour cells were used as a negative control. Cells grown in TCS broth at 30°C for 12 hours contained maximum precursor, and therefore these cells were used as positive precursor cells.

To establish optimum conditions for glutaraldehyde fixation of the cells, two criteria were set:

1. The preservation of the integrity of the cells

Figure 4. Elution Profile of Conjugated Ferritin-IgG on Sepharose 6B.

Conjugation procedure of ferritin-IgG was as described in Chapter II. Two major peaks were obtained on Sepharose 6B. Peak I represents large molecular weight aggregates of ferritin-IgG and Peak II represents unconjugated ferritin. The shoulder (arrow) represents a 1:1 ratio of ferritin:IgG [71]. Tubes were collected from the shoulder to be used as conjugate for electron microscopy.

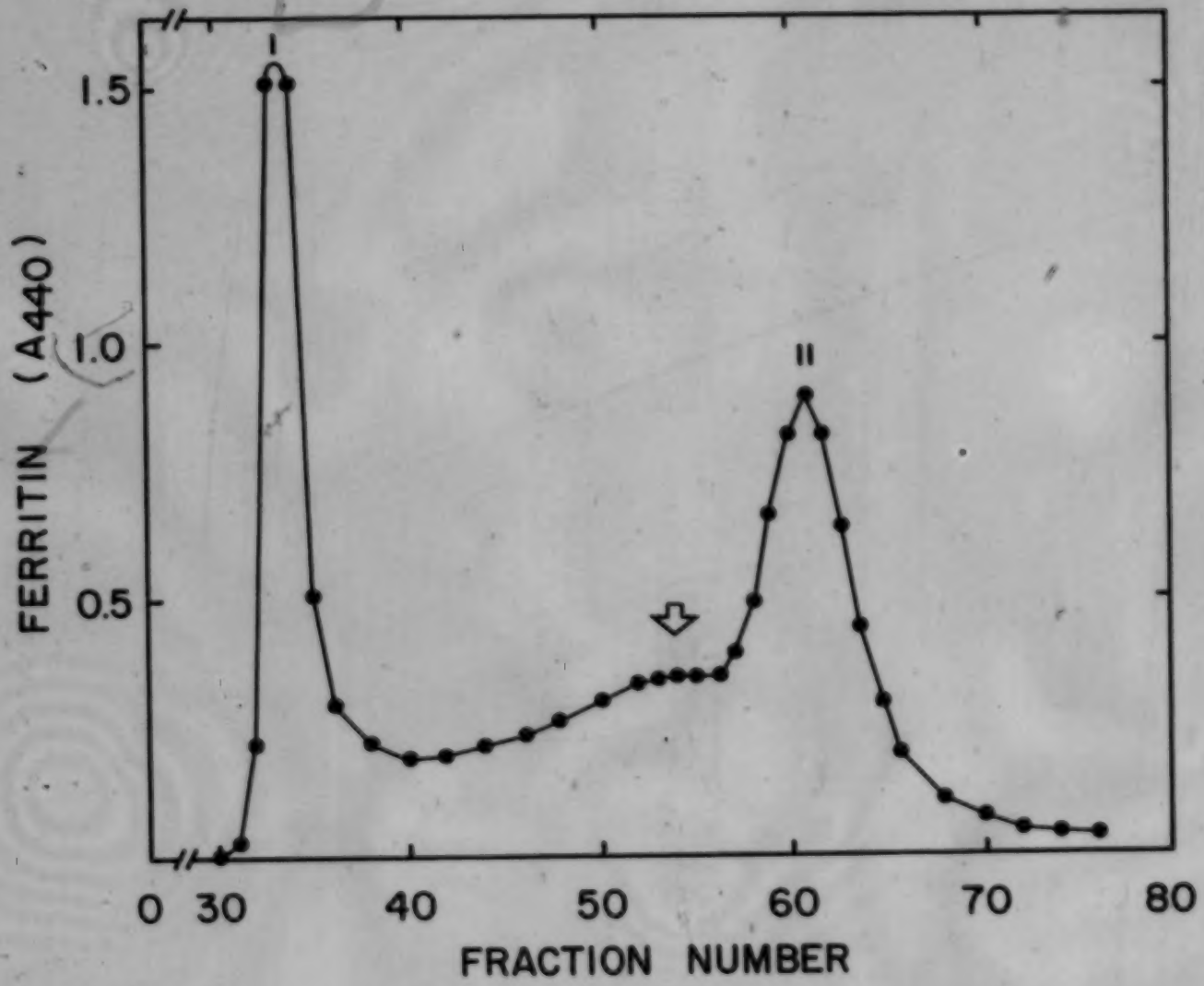

I
II
1.5
1.0
0.5
FERRITIN (A440)
0
30
40
50
60
70
80
FRACTION NUMBER

PLATE III Ouchterlony double diffusion plate showing precursor-negative control cells.

Well 1 = Pure protease 1

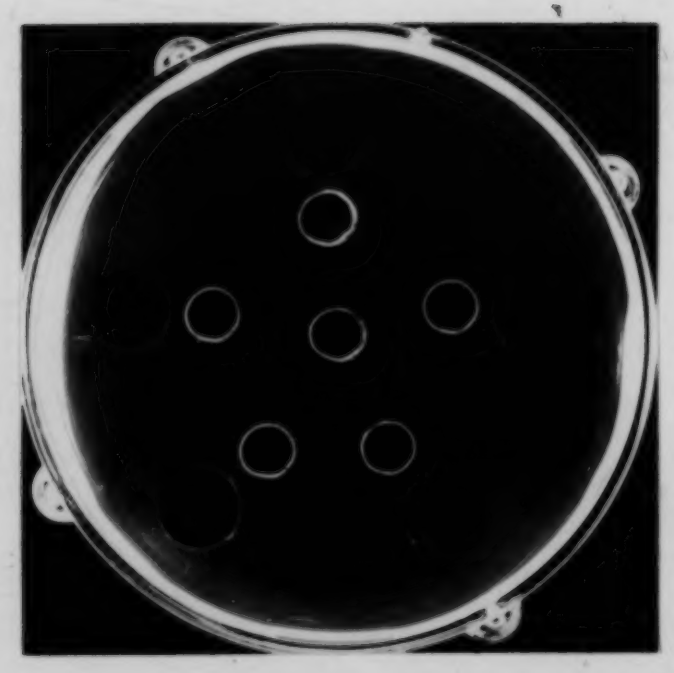
Well 2 = Crude cell extract from 12 hour cells grown at 30°C

Well 3 = Culture supernatant from 12 hour cells grown at 30°C

Well 4 = Crude cell extract from 24 hour cells grown at 37°C

Well 5 = Culture supernatant from 24 hour cells grown at 37°C

Centre well = Purified anti-protease 1 IgG



so that they will retain their shape in frozen thin sections.

2. The retention of 5-10% of the activity of the enzyme in order to ensure preservation of a degree of original antigenicity of the enzyme (J.W. Costerton, personal communication).

1% glutaraldehyde was initially used to fix *P. aeruginosa* cells (2 hours at 4°C). Good thin sections were obtained but no precursor activity could be detected. A ten-fold lower concentration of glutaraldehyde yielded broken cells and cell debris rather than intact cells on thin sectioning. To find a more optimum concentration of glutaraldehyde, various glutaraldehyde concentrations were tried and Table IV shows the results on the retention of precursor activity. The best concentration was 0.2% glutaraldehyde which resulted in 22% retention of precursor activity. The length of time of fixation using 0.2% glutaraldehyde was also varied to ensure proper fixing of the cells. Table V shows that when cells were fixed for 6 hours or longer, 10% of the precursor activity remained and cell integrity was also retained upon thin sectioning (Plate IV). Notice the lack of cytological detail in frozen thin sections of unstained cells.

Table IV

Precursor Activity in Whole Cells vs Concentration of Glutaraldehyde

Concentration (%) of[a.] Glutaraldehyde	Precursor[b.] (units/ml whole cells)	% Retention of Activity
0	12.4	100
1.0	0	0
0.75	0	0
0.50	0	0
0.40	0	0
0.30	0.222	1.8
0.20	2.8	22.6
0.10	11.0	88.7

a. Cells were fixed for 2 hours at $4^{o}C$

b. Whole cells were treated with toluene before assaying for precursor

Table V

Precursor Activity in Whole Cells vs Fixation Time

Fixation Time[a.] (hr)	Precursor[b.] (units/ml whole cells)	% Retention of Activity
0	10.1	100
2	1.87	18.5
4	1.58	15.6
5	1.31	13.0
6	1.03	10.2
24	1.04	10.3

a. Cells were fixed in 0.2% glutaraldehyde at $4^{o}C$

b. Whole cells were treated with toluene before assaying for precursor

PLATE IV Frozen thin sections of 12 hour *P. aeruginosa* 34362A cells, unstained.

Cells were fixed in 0.2% glutaraldehyde for 16 hours as described in Chapter II. The cells were sectioned and viewed under the electron microscope to confirm the presence of intact cells.

Bar on this micrograph represents 0.5 um.

As a result, the procedure of fixing in 0.2% glutaraldehyde for 14 hours at 4°C was used routinely.

(d) Staining frozen thin sections with IgG-Ferritin Conjugates: Frozen thin sections of 12 hours cells, containing precursor, were examined to determine the location of the precursor within the cell. Sections were stained with ferritin-anti-protease 1 conjugate. A definite region of concentration of label is seen at the periphery of the cells, while the cytoplasm is only sparsely labelled (Plate V). When sections from the same 12 hours cells were stained with ferritin-normal IgG conjugate, no ferritin granules could be seen (Plate VI, *A*) which eliminated the possibility of non-specific binding of the conjugate. Sections of precursor-negative cells (24 hr,37°C) exposed to the ferritin-anti-protease 1 conjugate, likewise showed no ferritin concentration above the background levels (Plate VI, *B*).

II. DISCUSSION

The results presented here indicate the presence of a cell-associated protein which is serologically related to extracellular protease 1 of *P. aeruginosa* 34362A. This protein is enzymatically inactive in the cell extract, but can be rendered active by reacting the extract with another

PLATE V Frozen thin sections of *P. aeruginosa* 34362A, positive for precursor, and exposed to a ferritin-anti-protease 1 conjugate.

Cells show heavy reactivity at their periphery, while the cytoplasm shows light labelling with ferritin.

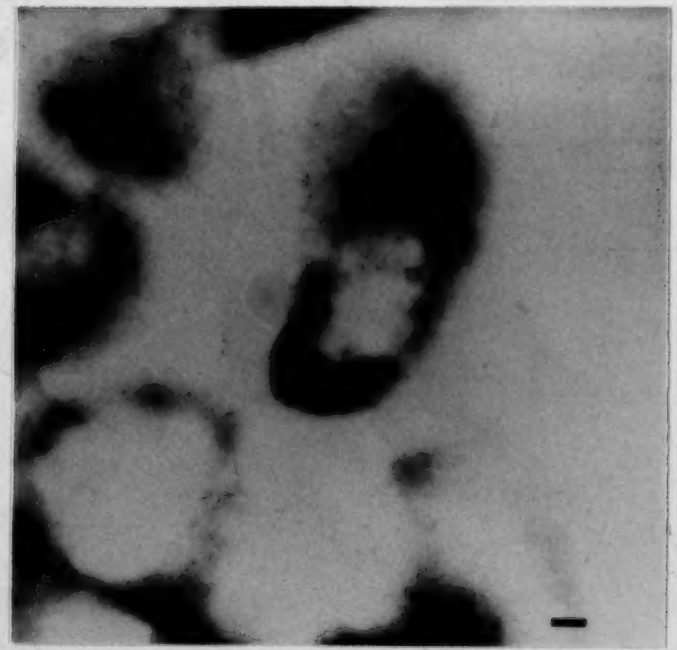
Bar on micrograph represents 0.4 um.

PLATE VI *A*: Cells of *P. aeruginosa* 34362A,negative for precursor, and exposed to a ferritin-anti-protease 1 conjugate.

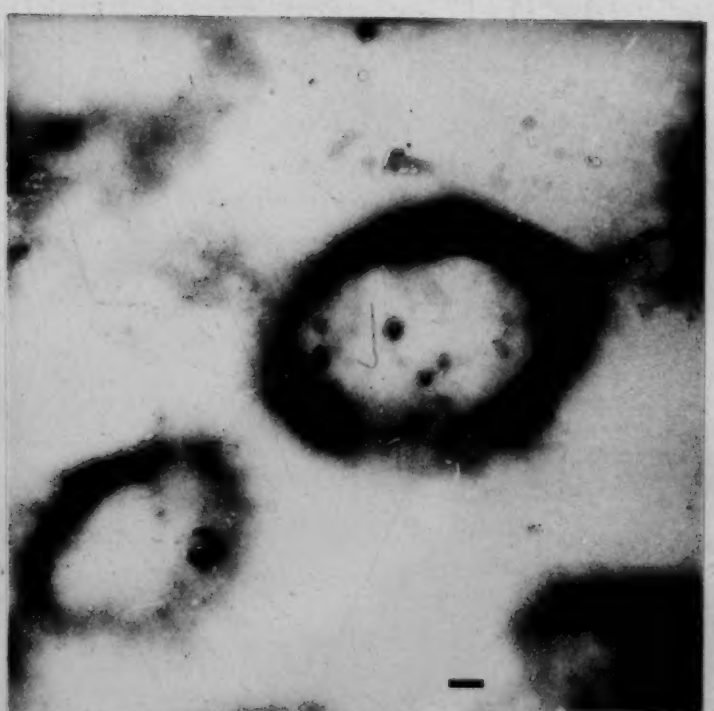
B: Cells of *P. aeruginosa* 34362A, positive for precursor, and exposed to a non-specific conjugate.

Bars on micrographs represent 0.2 um.

A



B



protease, namely protease 2. It has been shown by Jensen *et al.* [64] that the activatable protein in cell extract is the same protein that is serologically related to protease 1 (*i.e.*, precursor). These workers showed that the activatable material could be specifically removed from the cell extract by passage through an immunoabsorbance column specific for protease 1 but not one specific for alkaline phosphatase. The activatable material (precursor) was speculated to represent a normal intermediate stage in the synthesis of extracellular protease 1.

Growth studies showed that the appearance of precursor in the cell extract occurred at the same time as the rapid expulsion of extracellular protease 1 when cells were grown in TCS broth. Accumulation of precursor occured for 12 hr, after which there was a decline, accompanied by a further increase in the accumulation of extracellular protease 1. These data suggest that precursor may by involved as an intermediate in the secretion of protease 1.

It was important to determine the location of precursor in order to determine its role in the secretion of protease 1. Biochemical localization procedures (cell fractionation) indicate that precursor is not cytoplasmic but rather is an envelope-associated (periplasmic) protein. However, precursor does not behave in the same manner as

classic periplasmic enzymes such as alkaline phosphatase since it cannot be released by extraction with 0.2 *M* $MgCl_2$. This is in agreement with the differential release of periplasmically located proteins previously noted by Bhatti *et al.* [78] and interpreted by them as representing different modes of association of the proteins in the periplasm.

Immunocytological localization of precursor using ferritin-labelled antibodies indicated that precursor is found in greater concentration at the periphery of the cell. These results, in conjunction with those of the biochemical localization, present a strong argument that the majority of the precursor is located outside of the cytoplasmic membrane.

CHAPTER IV

PURIFICATION OF CELL-ASSOCIATED PRECURSOR FROM CRUDE CELL LYSATE

I. INTRODUCTION

The presence of a cell-associated but enzymatically inactive precursor to exocellular protease 1 was established by experiments described in Chapter III. In order to characterize the precursor further, it was necessary to obtain a pure preparation of this protein. The following attempts at purification were made, but in each case it resulted in spontaneous activation of the precursor to active protease.

II. RESULTS

A. Ion Exchange Chromatography: A 10 ml sample of crude cell extract containing 37 units of precursor was applied to a pre-equilibrated DEAE-cellulose column (see Chap. II). The elution profile of such a column is shown in Fig. 5. It can be seen that precursor was eluted from the column before the addition of the NaCl gradient (Peak I). When Peak I was assayed for proteolytic activity it was found that the chromatographic purification resulted in >90% activation of

Figure 5. Elution Profile of Cell Lysate from DEAE-cellulose Using a NaCl Gradient.

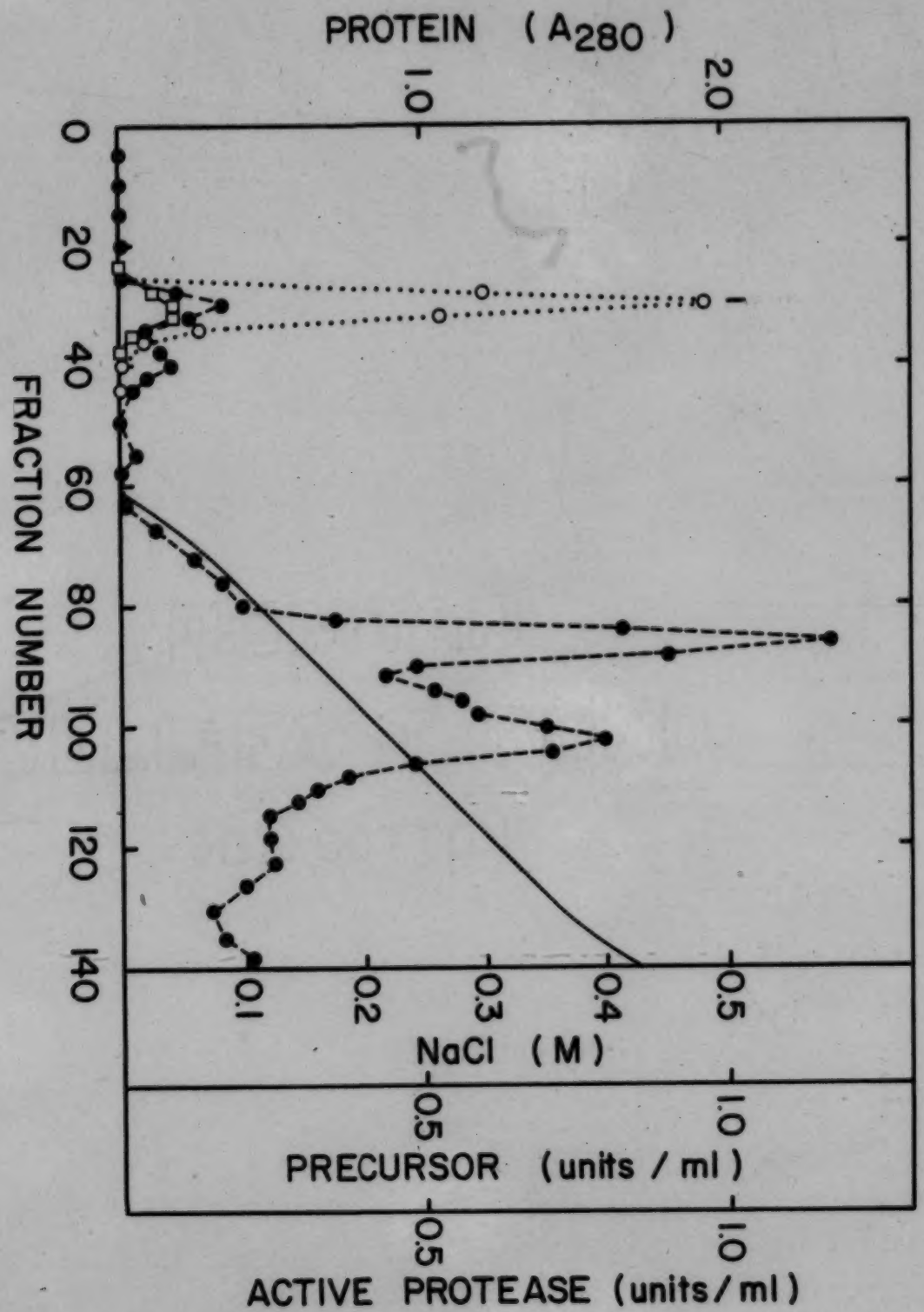
Chromatography was carried out as described in Chapter II. All fractions were assayed for precursor and protease activity, UV absorbance, and NaCl concentration.

Protein (A_{280}): ●——●

Protease activity (units/ml): O····O

Precursor activity (units/ml): □——□

NaCl (*M*): ——


PROTEIN (A280)
1.0
2.0
0
20
40
60
80
100
120
140
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
NaCl (M)
0.5
1.0
PRECURSOR (units / ml)
0.5
1.0
ACTIVE PROTEASE (units/ml)

the precursor.

B. Gel Filtration: Inorder to see if the activation of precursor on DEAE-cellulose might be a peculiarity of the ion exchange system, a 5 ml sample of crude cell extract (14 units of precursor) was chromatographed on a pre-equilibrated Sephadex G-100 column (see Chap. II). The elution profile from this column is shown in Fig. 6. Again, the precursor was essentially totally converted to active protease 1 (Peak I). It therefore appears that either purification procedure will result in the spontaneous activation of precursor to active protease 1.

C. Ammonium Sulfate Precipitation of Crude Cell Lysate: It was found that precursor could be concentrated from crude cell extract by suspending the fraction precipitating between 35 and 55% $(NH_4)_2SO_4$. Recovery of 70% of the precursor with a 1- to 2-fold increase in specific activity resulted. Precursor remained inactive, but activatible for >1 year if $(NH_4)_2SO_4$ was present. However, when the precursor fraction was dialyzed overnight at 4°C against 0.01 *M* Tris-HCl, pH 7.5, 85-100% activation resulted. On the other hand, dialysis of crude cell extract overnight under the same conditions did not result in activation of the precursor. However, if dialysis was continued for at least 3 days with frequent changes of dialysis buffer, significant

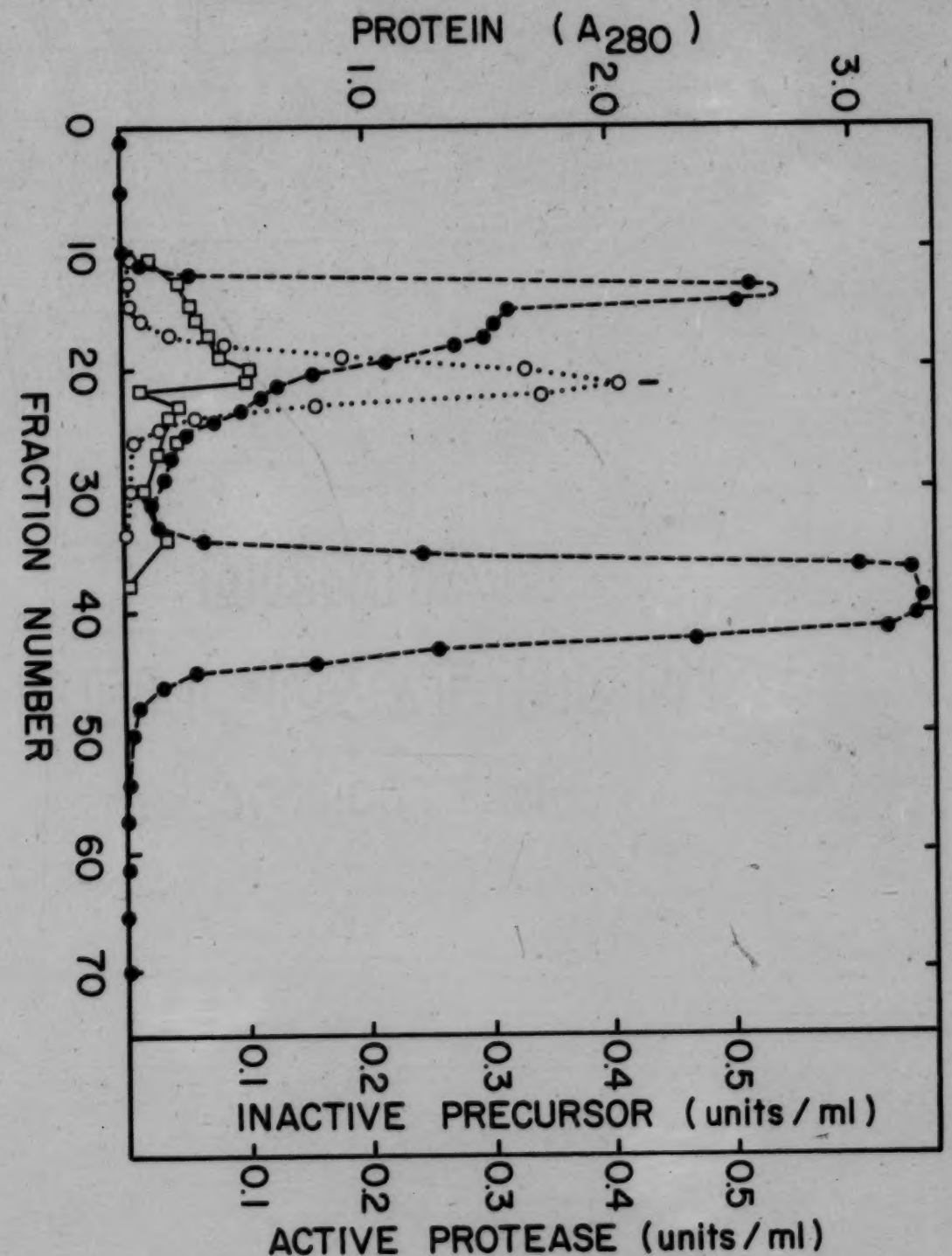
Figure 6. Purification of Precursor from Crude Cell Lysate by Gel Filtration (Sephadex G-100).

Sephadex G-100 chromatography was performed as described in Chapter II. All fractions were measured for UV absorbance and precursor and protease activity.

Protein (A_{280}): ●--●

Protease activity (units/ml): O····O

Precursor activity (units/ml): □——□


PROTEIN (A_{280})
1.0
2.0
3.0
0
10
20
30
40
50
60
70
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
INACTIVE PRECURSOR (units / ml)
0.1
0.2
0.3
0.4
0.5
ACTIVE PROTEASE (units / ml)

activation of precursor resulted. Conditions were examined which would prevent or induce activation of precursor from an $(NH_4)_2SO_4$ precipitate upon dialysis:

(i) Effect of $MgCl_2$ concentration on precursor activation: Samples of an $(NH_4)_2SO_4$ fraction of crude precursor were dialyzed against various concentrations of $MgCl_2$. Figure 7 shows that dialysis against 0.25 *M* $MgCl_2$ resulted in only 57% activation of precursor and that at 0.5 *M* $MgCl_2$ only 4% of the precursor became active. Therefore, further purification steps were performed using 0.5 *M* $MgCl_2$ in the hope of purifying precursor in its inactive state.

(ii) Effect of pH on precursor activation: Samples of an $(NH_4)_2SO_4$ fraction of crude precursor were dialyzed against various buffers ranging in pH from 4 to 8 to determine the effect of pH on activation of precursor. Fig. 8 shows that less than 10% activation occurred between pH 4 and 6.2. At pH 7 and above, at least 80% resulted. It should be noted that at pH 4 to 5.2 protein precipitation was observed but precursor activity was still retained when resuspended samples were activated with protease 2 under normal assay conditions. Therefore, pH 5.6 was used in the following attempts at purification of precursor in its inactive state under low pH conditions.

Figure 7. Effect of [$MgCl_2$] on the Stability of Precursor Upon Dialysis.

1 ml samples of an ammonium sulfate fraction of cell extract were dialyzed overnight at 4°C against various concentrations of $MgCl_2$ in 0.01 M Tris-HCl, pH 7.5. After dialysis, samples were assayed for both precursor and protease activity. An undialyzed sample of the above was also incubated at 4°C overnight and was used as a control (0% activation).

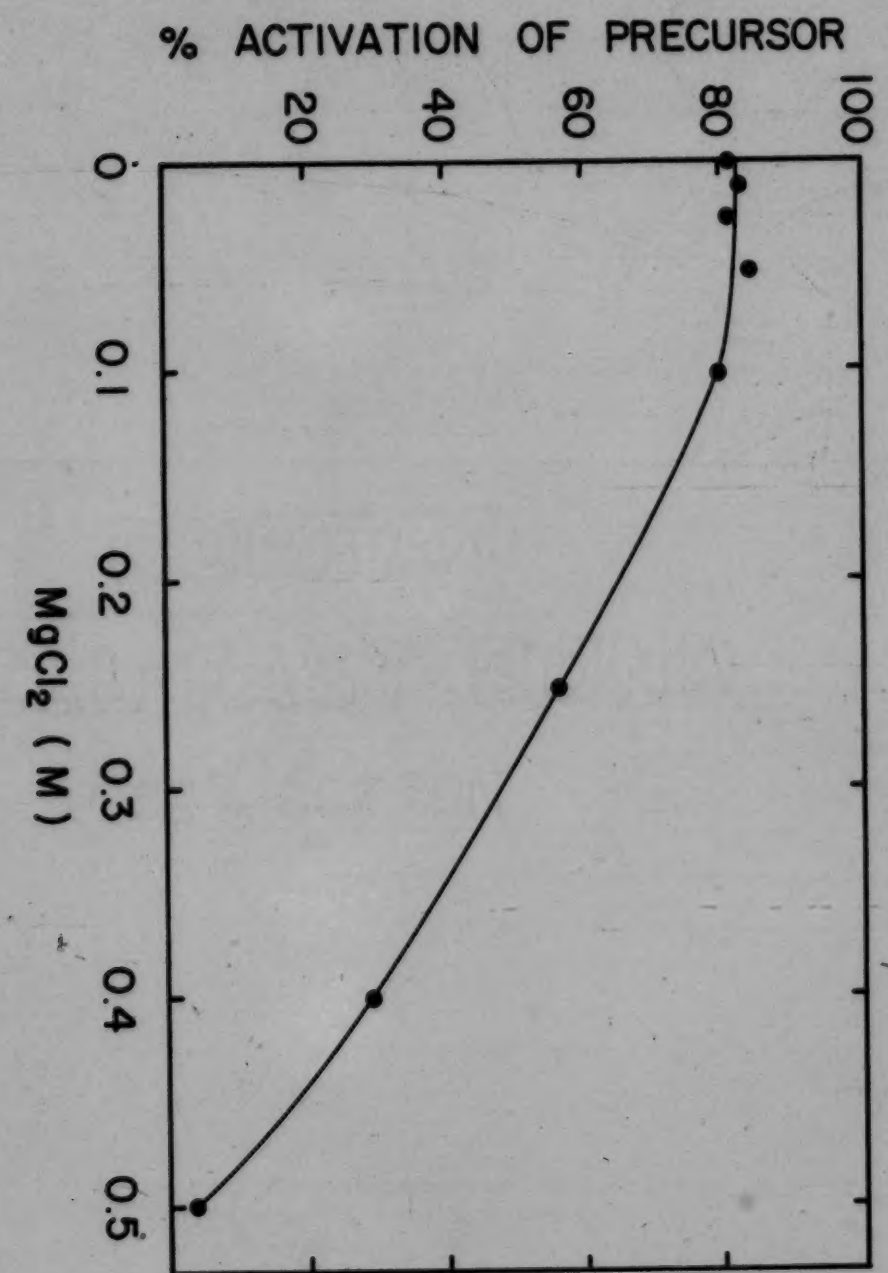
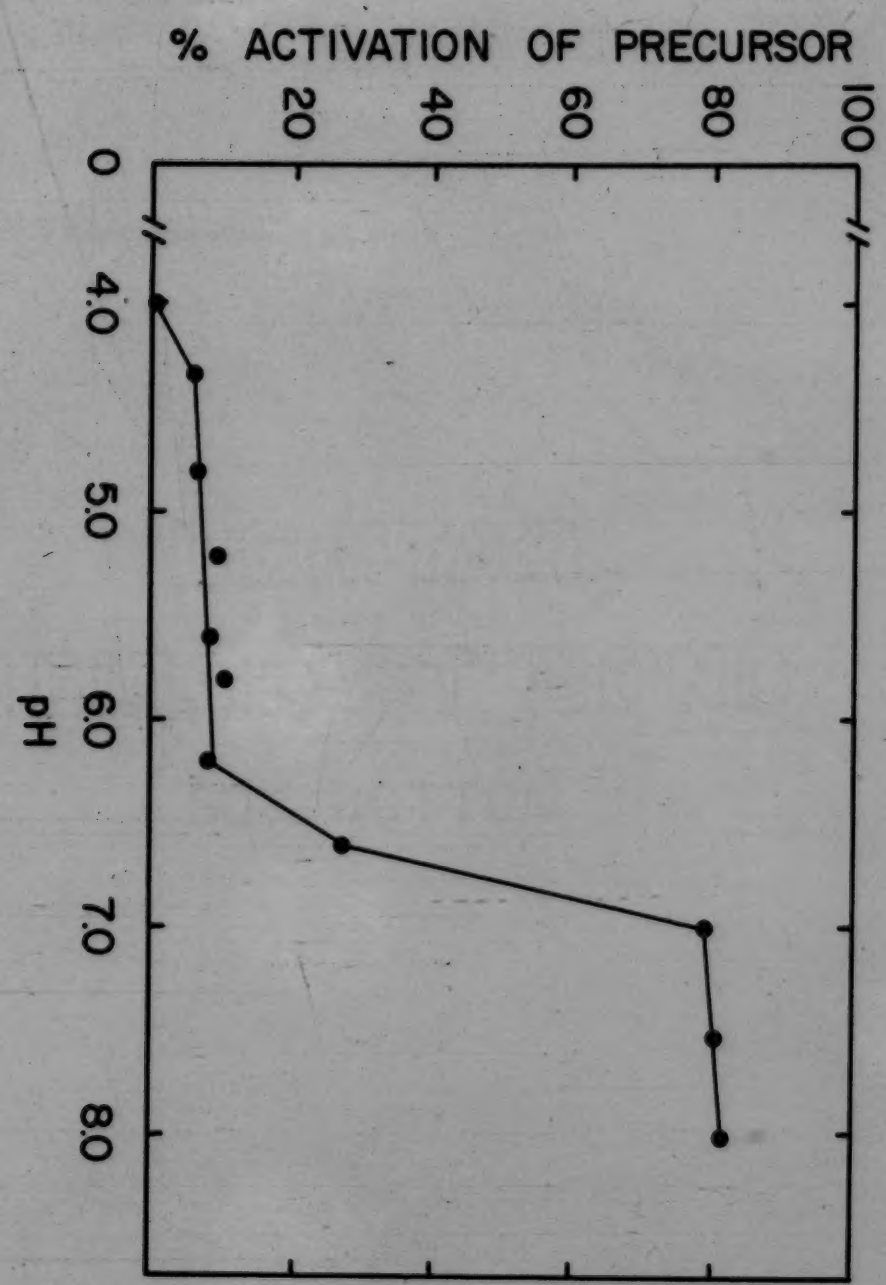

% ACTIVATION OF PRECURSOR
20
40
60
80
100
0
0.1
0.2
0.3
0.4
0.5
MgCl2 (M)

Figure 8. Effect of pH on the Stability of Precursor Upon Dialysis.

1 ml samples of an $(NH_4)_2SO_4$ fraction of cell extract were dialyzed overnight at 4°C against buffers of different pH. 0.01 *M* sodium acetate-acetic acid buffer was used for pH 4-5.6, and 0.01 *M* sodium phosphate was used for pH 5.8-8.0. After dialysis, samples were assayed for precursor and protease activity. An undialyzed sample of the above was also incubated under the same conditions and was used as a 0% activation control.


% ACTIVATION OF PRECURSOR
20
40
60
80
100
0
4.0
5.0
6.0
7.0
8.0
pH

(iii) Dialysis of $(NH_4)_2SO_4$ fractions of precursor against other buffers: Samples of an $(NH_4)_2SO_4$ fraction were dialyzed against a variety of other buffers. Table VI lists the dialysis buffers tested and the per cent activation measured after 16 hours. It can be seen that activation results from dialysis against neutral buffers, but is greatly reduced by low pH or high ionic strength.

D. Attempts to Purify Precursor under Stabilizing Conditions:

(i) Sephadex G-100 Chromatography in 0.5 *M* $MgCl_2$: An $(NH_4)_2SO_4$ fraction of crude precursor was dialyzed against 0.01 *M* Tris-HCl, 0.5 *M* $MgCl_2$, pH 7.5 and was chromatographed on Sephadex G-100 pre-equilibrated with the same buffer. Essentially no inactive precursor was recovered (Fig. 9); the majority was recovered as active protease (Peak I). Therefore, although the buffer containing high $MgCl_2$ concentration may stabilize precursor when dialyzed, it does not have the equivalent protective effect during purification by Sephadex G-100.

(ii) CM-Cellulose chromatography at pH 5.6: In order to determine whether low pH would allow purification of precursor in its inactive state, the following purification was attempted. An $(NH_4)_2SO_4$ fraction of crude precursor was dialyzed against 0.01 *M* sodium acetate and chromatographed on a CM-cellulose column using a linear

Table VI

Activation of Precursor by Dialysis
Against Various Buffers

Dialysis Buffer	% Activation
0.01 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.5	88
0.01 M sodium phosphate, pH 7.5	81
distilled H_2O	20
0.01 M sodium acetate, pH 5.6	9
0.01 M sodium phosphate, pH 6.2	8
0.01 M Tris-HCl, 0.5 M $MgCl_2$, pH 7.5	4
0.01 M Tris-HCl, 0.5 M $CaCl_2$, pH 7.5	0
0.01 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$, pH 7.5	0

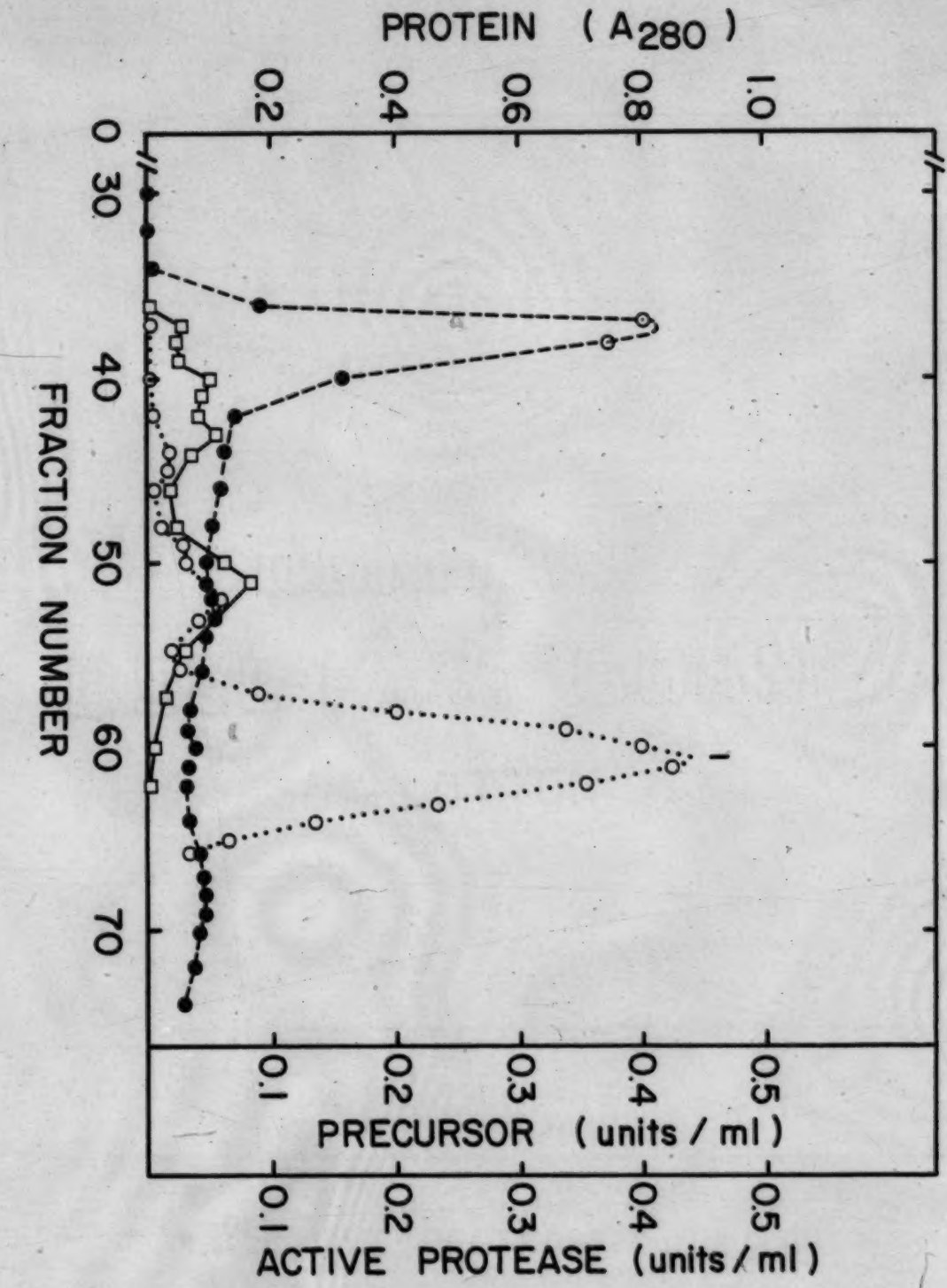
Figure 9. Gel Filtration (Sephadex G-100) in 0.5 *M* $MgCl_2$ of an Ammonium Sulfate Fraction of Crude Cell Lysate.

Sephadex G-100 chromatography was carried out in 0.01 *M* Tris-HCl, 0.5 *M* $MgCl_2$, pH 7.5 as described in Chapter II. All fractions were measured for UV absorbance and precursor and protease activity.

Protein (A_{280}): ●——●

Protease activity (units/ml): O····O

Precursor activity (units/ml): □——□


PROTEIN (A280)
0.2
0.4
0.6
0.8
1.0
0 30 40 50 60 70
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
PRECURSOR (units / ml)
0.1
0.2
0.3
0.4
0.5
ACTIVE PROTEASE (units / ml)

gradient of NaCl to elute adsorbed proteins. The major peak of precursor (Peak I, Fig. 10) was recovered as active protease. A smaller peak (Peak II), which eluted at higher NaCl concentration than active precursor, was inactive precursor. When Peak II fractions were pooled and reassayed, 100% activation had occurred, presumably due to the residual protease activity in this region.

(iii) Sephadex G-100 chromatography at pH 5.6: An $(NH_4)_2SO_4$ fraction of crude precursor was dialyzed against 0.01 *M* sodium acetate, pH 5.6 and was applied to a Sephadex G-100 column. In this case, very little protein separation occurred (Fig. 11); the majority of the protein, including precursor, was eluted as a single peak close to the void volume. Precursor remained inactive but no purification (*i.e.*, increase in specific activity) was achieved. It appeared that protein aggregation had occurred under these conditions which protected the precursor from activation. This protein aggregation could be due to the low molar concentration of the buffer used.

E. Analyses for the Presence of an Endogenous Inhibitor Moiety of Precursor: The fact that precursor remained inactive in crude cell extract, but activated upon purification (*eg.*, column chromatography), suggested that the removal of an inhibitor molecule might be the cause the activation. Therefore, eluted fractions from DEAE-cellulose

Figure 10. CM-cellulose Chromatography of an Ammonium Sulfate Fraction of Crude Cell Lysate.

CM-cellulose chromatography was carried out under low pH conditions as described in Chapter II. All fractions were assayed for precursor and protease activity, UV absorbance, and NaCl concentration.

Protein (A_{280}): ●---●

Protease activity (units/ml): O····O

Precursor activity (units/ml): □——□

NaCl (*M*): ——

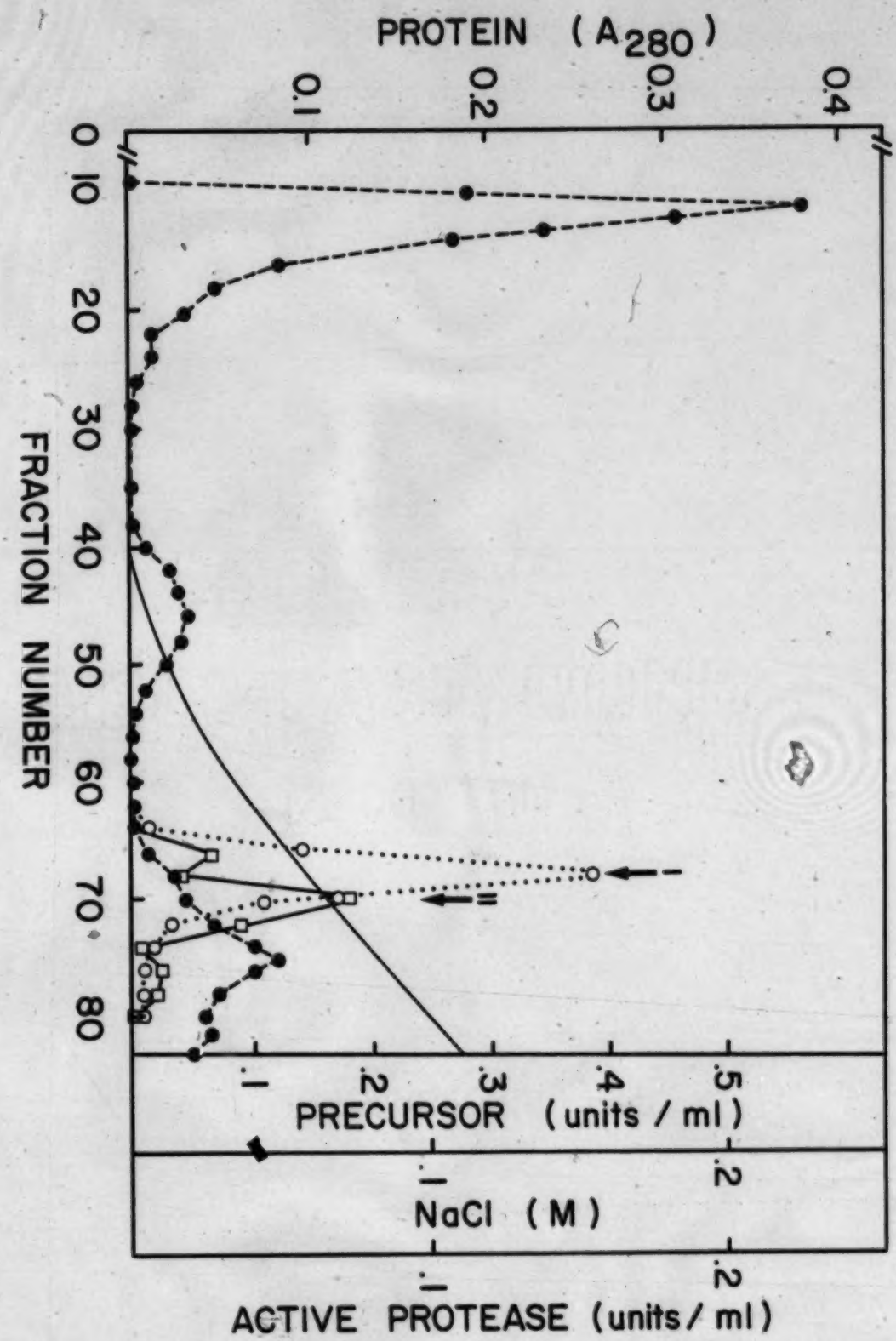

PROTEIN (A280)
0.1
0.2
0.3
0.4
0 10 20 30 40 50 60 70 80
FRACTION NUMBER
I
II
.1 .2 .3 .4 .5
PRECURSOR (units / ml)
.1 .2
NaCl (M)
.1 .2
ACTIVE PROTEASE (units / ml)

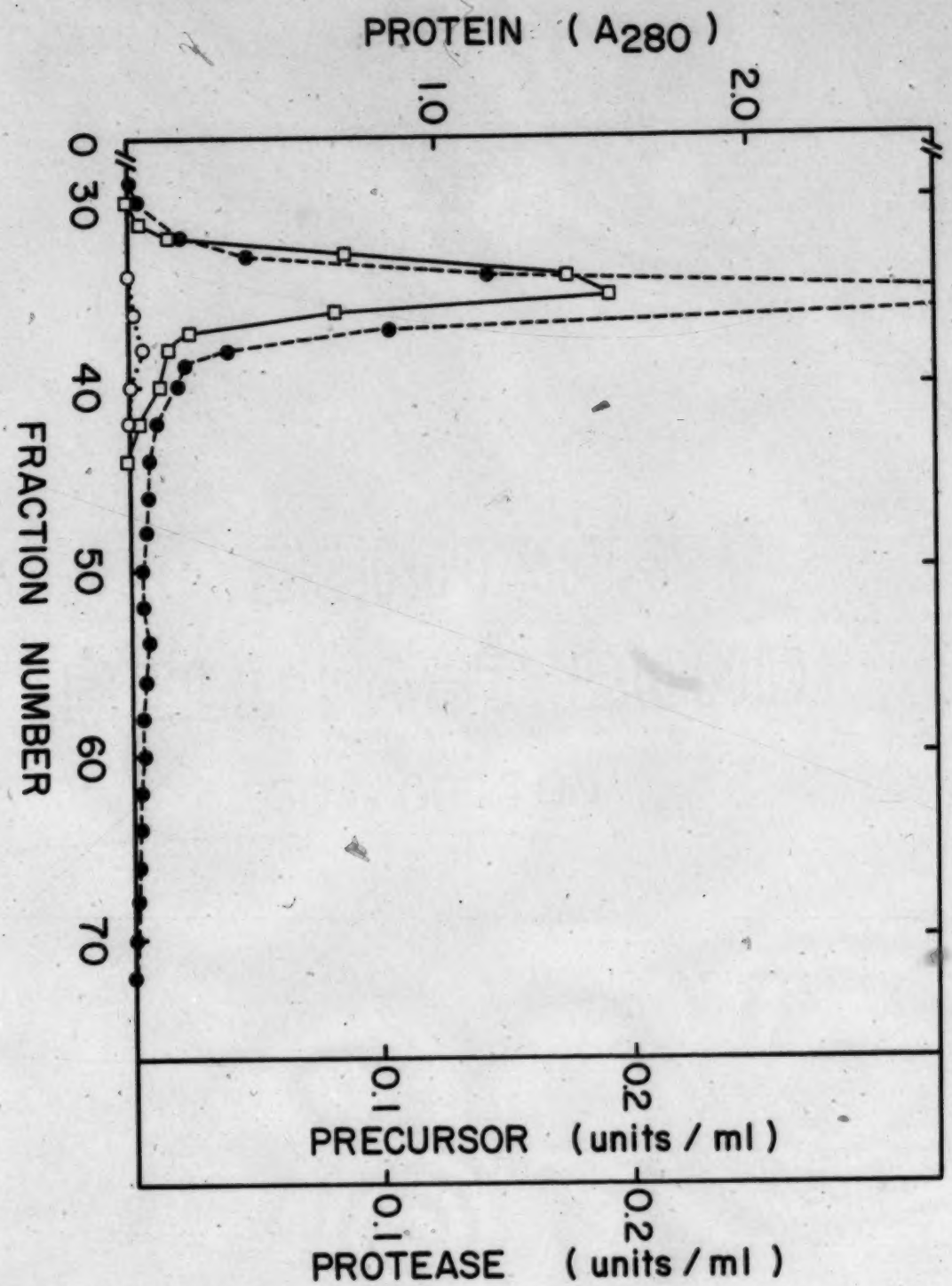
Figure 11. Gel Filtration (Sephadex G-100) in 0.01 *M* Sodium Acetate, pH 5.6 of an Ammonium Sulfate Fraction of Crude Cell Lysate.

Sephadex G-100 chromatography was carried out as described in Chapter II. All fractions were measured for UV absorbance and precursor and protease activity.

Protein ($A_{...}$): ●---●

Protease activity (units/ml): O····O

Precursor activity (units/ml): □——□


PROTEIN (A280)
1.0
2.0
0
30
40
50
60
70
FRACTION NUMBER
0.1
0.2
PRECURSOR (units / ml)
0.1
0.2
PROTEASE (units / ml)

and immunoabsorbance columns were analyzed for the presence of an endogenous inhibitor.

(i) DEAE-cellulose column: As previously shown (Fig. 5), precursor was eluted from the DEAE-cellulose column before the application of the NaCl gradient and was recovered as an active protease. Eluted, non-enzymatic fractions from the DEAE-cellulose column were pooled and equal volumes of activated precursor and pooled fractions were allowed to react for 1 hour at room temperature, after which they were assayed for decrease in proteolytic activity. None of the pooled fractions exhibited any inhibitory effect on the activated precursor. Therefore, if an inhibitor moiety were present, it did not recombine with the active precursor; activation of precursor appeared to be an irreversible process.

(ii) Immunoabsorbance column: Precursor is selectively adsorbed to an immunoabsorbance column specific for protease 1 (see Chap. V). When precursor is eluted from the column with 0.01 *N* NaOH it is recovered in an active state. If an inhibitor moiety were present, it would be expected to be washed through the column, allowing the precursor to be removed as an active protease. Equal volumes of activated precursor and effluent proteins were incubated at room temperature for 1 hour, after which the mixture was assayed for a decrease in proteolytic activity. No

inhibition of active precursor was observed.

III. DISCUSSION

The results presented in this Chapter indicate that precursor cannot be purified in its inactive state by the use of the various techniques pursued. Both DEAE-cellulose and Sephadex G-100 chromatography resulted in the spontaneos activation of precursor. Conditions for stabilizing precursor were investigated. It was found that a high ionic strength and low pH allowed the precursor to remain largely inactive upon dialysis. Therefore, columns were run using either 0.5 *M* MgCl, or sodium acetate, pH 5.6. All of these attempts failed to purify precursor in an inactive state.

The observation that both dialysis and purification resulted in the activation of precursor lead to the speculation that the removal of a specific inhibitor moiety may be responsible for activation. However, a search for such a simple inhibitor proved unsuccessful.

It appears that the precursor remains in its inactive (but activatable) state only when in association with other molecules, likely proteins, present in the crude cell extract. Removal of these contaminating molecules by whatever purification procedures results in irreversible activation of the precursor suggesting that some change in the precursor, which alters this association has occured. It

also suggests that attempts to study the problem of activation by first obtaining a purified inactive precursor for direct comparison with active enzyme would continue to be frustrating so a different approach had to be taken. This was to purify unactivated precursor under denaturing conditions to prevent possible autoproteolysis and to analyze the protein for differences in molecular weight or N-terminal amino acid from the native protein (see Chap. V). Further studies on the mechanism of precursor activation were performed using a crude cell extract containing unactivated precursor and are discussed in Chapter VI.

CHAPTER V

CHARACTERIZATION OF AN INACTIVE CELL-ASSOCIATED PRECURSOR OF AN EXOCELLULAR PROTEASE OF *Pseudomonas aeruginosa* 34362A

I. INTRODUCTION

In Chapter IV it was demonstrated that the precursor activated upon purification by conventional procedures. This Chapter deals with measurements of molecular weight and N-terminal residues made using crude precursor (with stabilizing proteins in place) or precursor isolated under denaturing conditions and comparison with values from purified active protease 1.

II. RESULTS AND DISCUSSION

A. Molecular Weight Determinations

(i) Glycerol Density Gradient Centrifugation: If the presence of a prepiece were responsible for rendering cell-associated precursor inactive, this would be reflected by a difference in molecular weight between precursor and active protease 1. To this end, the sedimentation velocity of precursor (in cell extract) and pure protease 1 in 5-25% glycerol gradients were compared. Since protease 1 itself can activate precursor (see Chapter VI), precursor

and protease 1 could not be run together on the same gradients. Therefore separate gradients had to be run for each. For precise comparison to be possible, it was necessary to run replicate samples of each until identical gradients for the two separate preparations were obtained. Fig. 12 shows two such identical gradients superimposed, where a difference of 2.2 fractions was found between the peaks of the precursor and the active enzyme. Replicate experiments established the range of the difference to be 2.2-2.5 fractions. The faster sedimentation velocity of the precursor suggests either that precursor may be larger than protease 1, or that there is a significant difference in shape. It should be noted that when the gradient fractions were assayed, the precursor had become activated during the course of the centrifugation.

For comparison, this experiment was repeated using cell extract which had been activated proteolytically with protease 2, or non-enzymatically activated with deoxycholate (see Chapter VI) before application to the gradients. As shown in Figs. 13 and 14, neither activated precursor preparation exhibited significant difference in its rate of sedimentation from purified exocellular protease 1, the maximum difference being 0.5 fractions.

Figure 12. Glycerol Density Gradient (5-25%) Centrifugation of Unactivated Precursor and Pure Protease 1.

Separate gradients containing unactivated precursor (3.28 mg cell extract) and protease 1 (0.158 mg pure protease 1) respectively were run for 24 hours as described in Chapter II. Identical linear gradients were obtained and are superimposed for comparison purposes.

Precursor activity (units/ml): ●····●

Protease 1 activity (units/ml): ■- - -■

Glycerol concentration (%): ——

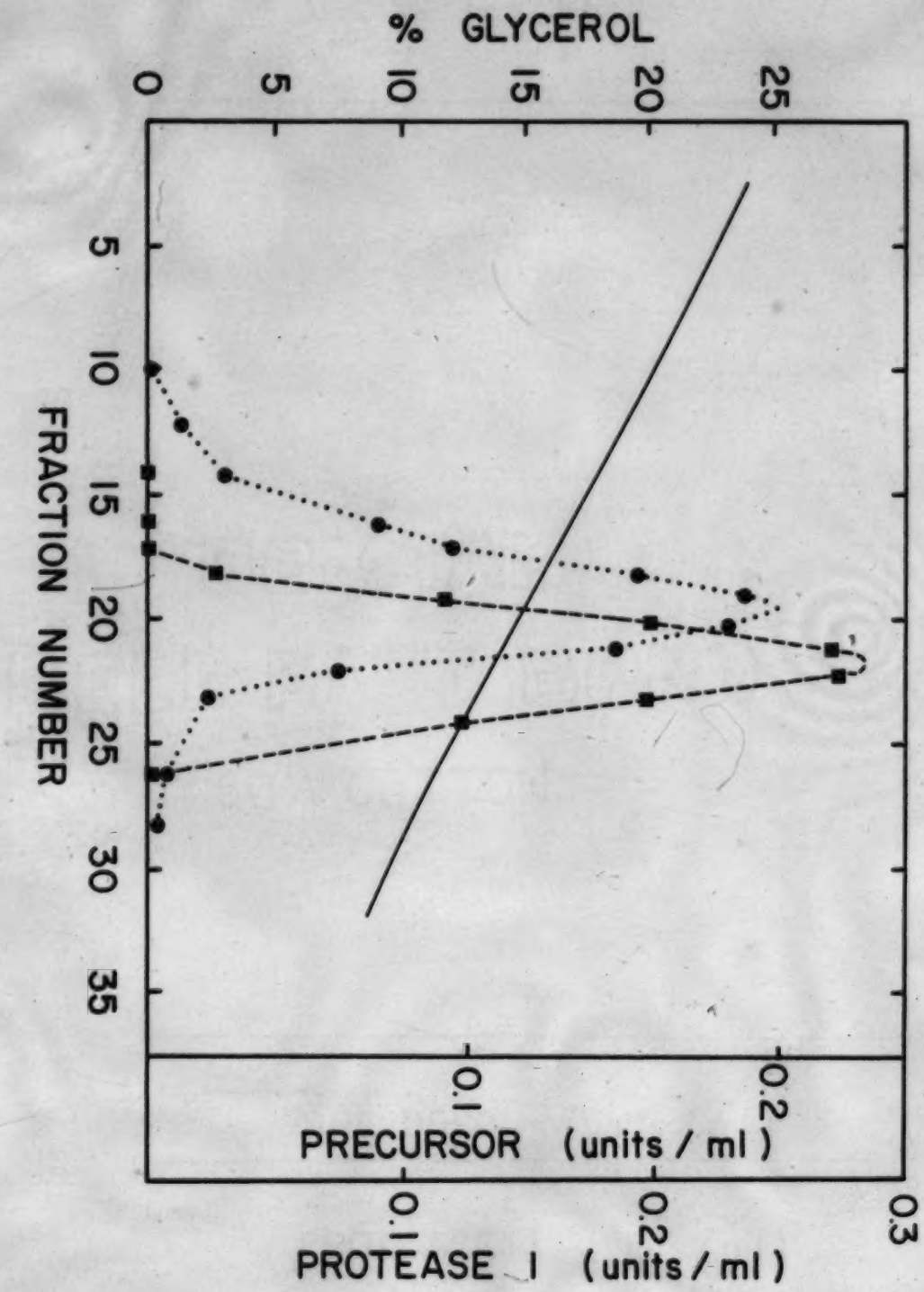

% GLYCEROL
0
5
10
15
20
25
FRACTION NUMBER
5
10
15
20
25
30
35
PRECURSOR (units / ml)
0.1
0.2
PROTEASE I (units / ml)
0.1
0.2
0.3

Figure 13. Glycerol Density Gradient (5-25%) Centrifugation of Protease 2 Activated Precursor and Pure Protease 1.

Separate gradients containing protease 2 activated precursor (3.28 mg cell extract) and protease 1 (0.177 mg pure protease 1) respectively were run for 24 hours as described in Chapter II. Identical linear gradients were obtained and are superimposed for comparison purposes.

Protease 2 activated precursor activity (units/ml): ●····●

Protease 1 activity (units/ml): ■-–-■

Glycerol concentration (%): ——

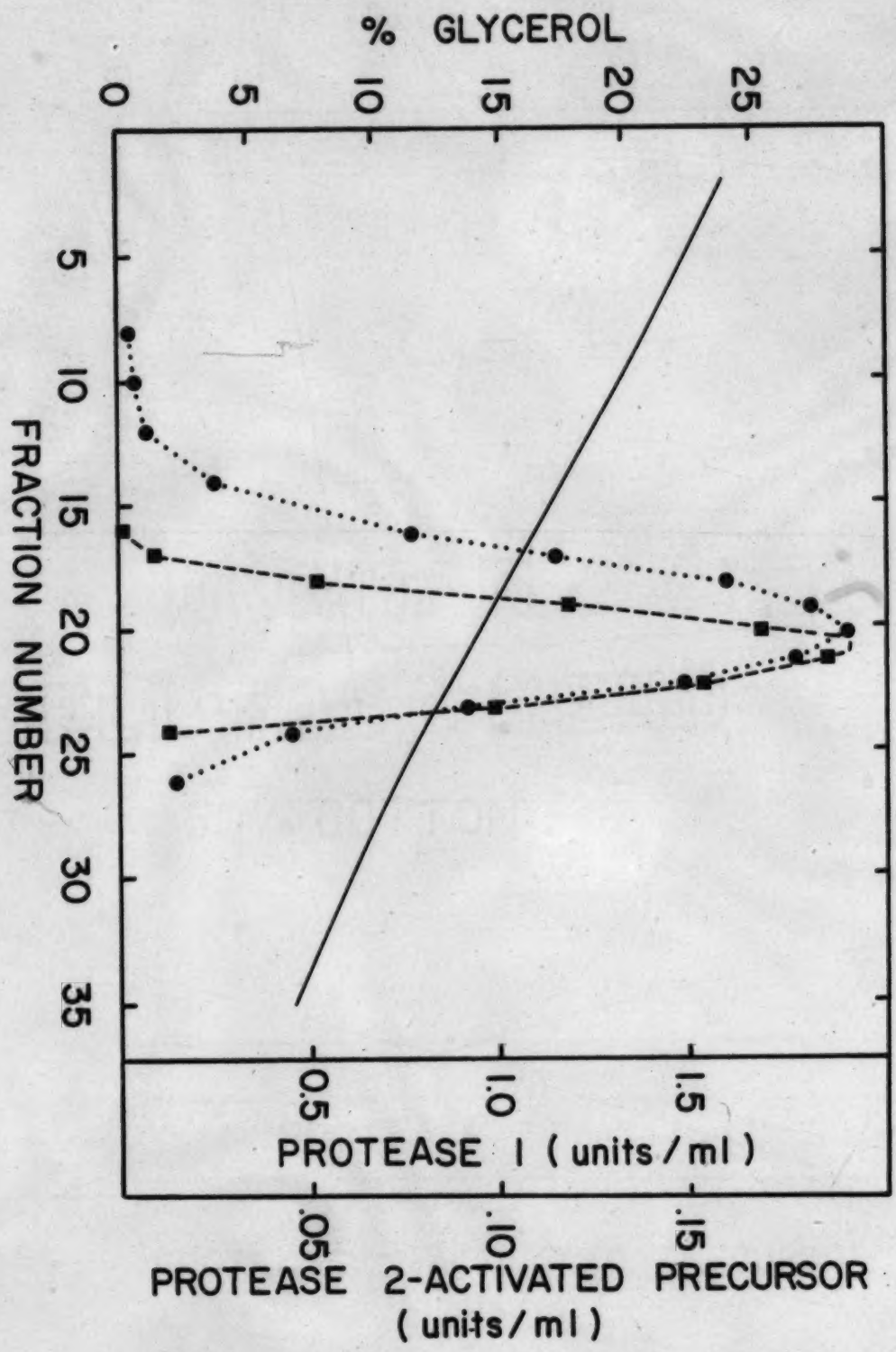

% GLYCEROL
0
5
10
15
20
25
FRACTION NUMBER
5
10
15
20
25
30
35
0.5
1.0
1.5
PROTEASE 1 (units / ml)
.05
.10
.15
PROTEASE 2-ACTIVATED PRECURSOR
(units / ml)

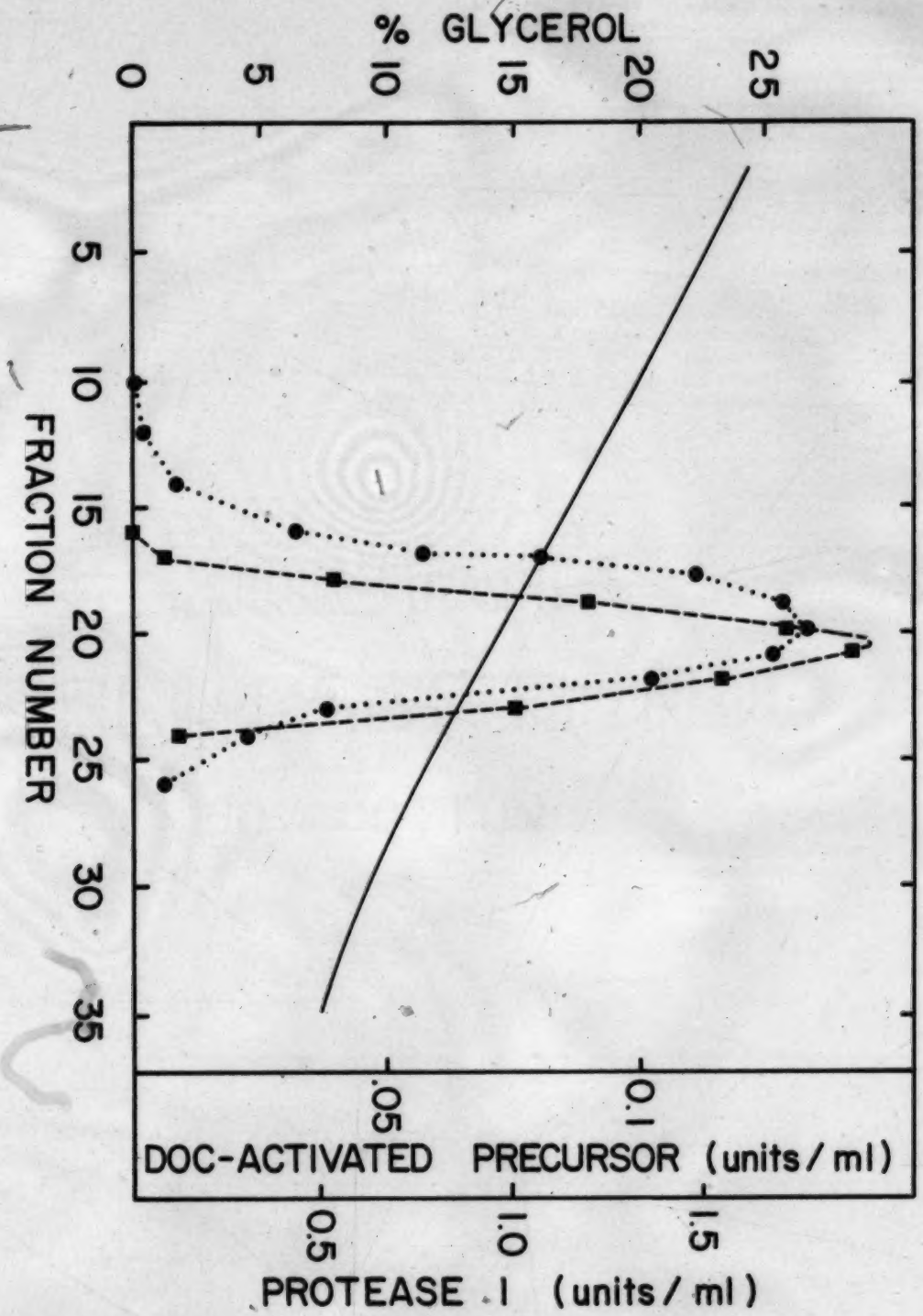
Figure 14. Glycerol Density Gradient (5-25%) Centrifugation of Deoxycholate Activated Precursor and Pure Protease 1.

Separate gradients containing deoxycholate activated precursor (3.28 mg cell extract) and protease 1 (0.177 mg pure protease 1) respectively were run for 24 hours as described in Chapter II. Identical linear gradients were obtained and are superimposed for comparison purposes.

Deoxycholate activated precursor activity (units/ml): ●····●

Protease 1 activity (units/ml): ■– –■

Glycerol concentration (%): ——

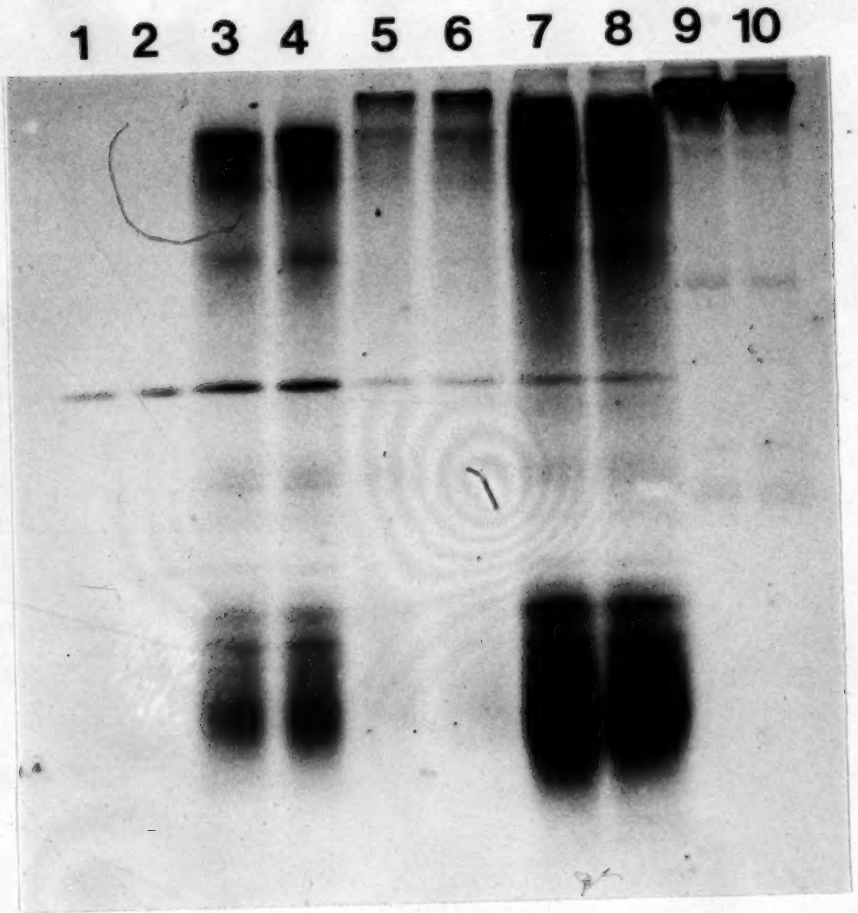

% GLYCEROL
0
5
10
15
20
25
FRACTION NUMBER
5
10
15
20
25
30
35
.05
.1
DOC-ACTIVATED PRECURSOR (units / ml)
0.5
1.0
1.5
PROTEASE I (units / ml)

The following experiments were designed to ascertain which of the two hypotheses, difference in molecular weight or difference in molecular configuration, best explain the observed difference in sedimentation velocity between unactivated precursor and activated enzyme.

(ii) Characterization of Immunoprecipitates of Precursor, Protease 2-activated Precursor and Protease 1: The possible difference in molecular weight was further investigated by means of immunoprecipitation of protease 1 and unactivated precursor and analysis of the immunoprecipitates by electrophoresis in 20% polyacrylamide in the presence of SDS. This technique has been shown to detect differences in molecular weight as little as 1000 [73]. Purified protease 1 was used as the reference protein. Washed immunoprecipitates of protease 1 from culture supernatant, precursor from cell extract, and protease 2-activated precursor from cell extract were prepared as described in Chapter II and subjected to electrophoresis. The results shown in Plate VII demonstrated that no significant difference in electrophoretic mobility could be detected between the three immunoprecipitates under these conditions. Protease 2 catalyzed activation of precursor gives rise to active protease having the identical molecular weight to that of the native enzyme. In spite of precautions taken to minimize proteolysis during immunoprecipitation, some

PLATE VII 20% polyacrylamide gel in SDS of immunoprecipitates.

Immunoprecipitates were prepared as described in Chapter II and run in duplicate on a 20% polyacrylamide gel in SDS. Tracks 1 & 2 contain purified protease 1 used as a reference protein, 3 & 4 contain immunoprecipitates of protease 1 from culture supernatant, 5,6 contain unactivated precursor from an $(NH_4)_2SO_4$ fraction of crude cell extract, 7,8 contain protease 2- activated precursor from an $(NH_4)_2SO_4$ fraction of crude cell extract, and 9,10 contain partially purified anti-protease 1 IgG.


1 2 3 4 5 6 7 8 9 10

autodigestion products, as well as digest products of IgG, could be detected as evidenced by the faint contaminating bands. These autodigestion products were also observed with the unactivated precursor, suggesting that activation of precursor has taken place. If an equivalent sample of crude unactivated precursor without antibody is incubated under identical conditions (*i.e.*, no immunocomplex formed), no activation of precursor occurs. These data suggest that there is no detectable molecular weight difference between unactivated precursor, activated precursor and active protease 1. However, they are not, in themselves, sufficient to eliminate the hypothesis that activation occurs by proteolytic cleavage of a signal peptide from the precursor during the formation of the immunoprecipitate. It has been long known that the formation of the antibody-antigen complex can affect conformation of polypeptide antigens [79,80] and even can activate "defective" enzymes [81,82]. It is possible that the formation of the precursor-antibody complex alters the conformation of the precursor, exposing its active site, resulting in autocleavage of the putative signal peptide. The presence of contaminating low molecular weight bands in all samples suggests that some auto-proteolysis does in fact occur in the immune complex.

B. Purification of Precursor under Denaturing Conditions: Clearly, the inherent problem of possible auto-activation of precursor to active protease complicates interpretation of molecular weight and N-terminal analyses. To overcome this it was decided to follow an alternative route, namely to subject both unactivated precursor and its active counterparts to irreversible denaturation, and to purify and compare the denatured (and thus unactivated) components, thereby eliminating the possibility of autoproteolysis. Immunoabsorbance chromatography (at 4°C) was used because of its high specificity. Samples of precursor from cell extract, protease 2-activated precursor from cell extract, and protease 1 from culture supernatant were applied to an immunoabsorbance column prepared with anti-protease 1 IgG (Figs. 15-17) The column was washed with 0.05 *M* Tris-HCl, pH 7.5 for 2 hours to remove the non-adsorbed contaminating protein (Panel A of Figs. 15-17) followed by elution with 5% formic acid to remove additional non-specifically bound proteins (Panel B). This non-specific material was shown by polyacrylamide gel electrophoresis in the presence of SDS to be unrelated to either the precursor or enzyme. Furthermore, the 5% formic acid serves to denature the remaining adsorbed protein, hence eliminating the problem of autodigestion. The adsorbed antigens were eluted from the column with 0.03 *N* NaOH and the fractions

Figure 15. Purification of Protease 1 by Immunoabsorbance Chromatography Under Denaturing Conditions.

A. 7.5 ml culture supernatant containing 44.4 units of protease were applied to a 28x4 cm immunoabsorbance column. Non-specific protein was eluted with 0.01 *M* Tris-HCl, pH 7.5, 5 ml fractions were collected, and the A_{280} of each was measured.

B. Non-specific protein was further removed by elution with 5% formic acid, 5 ml fractions were collected, and the A_{280} was measured for each fraction.

C. Specifically bound protein was recovered by elution with 0.03 N NaOH and 2.5 ml fractions were collected into tubes containing 2.5 ml 10% formic acid. The A_{280} was measured for each fraction.

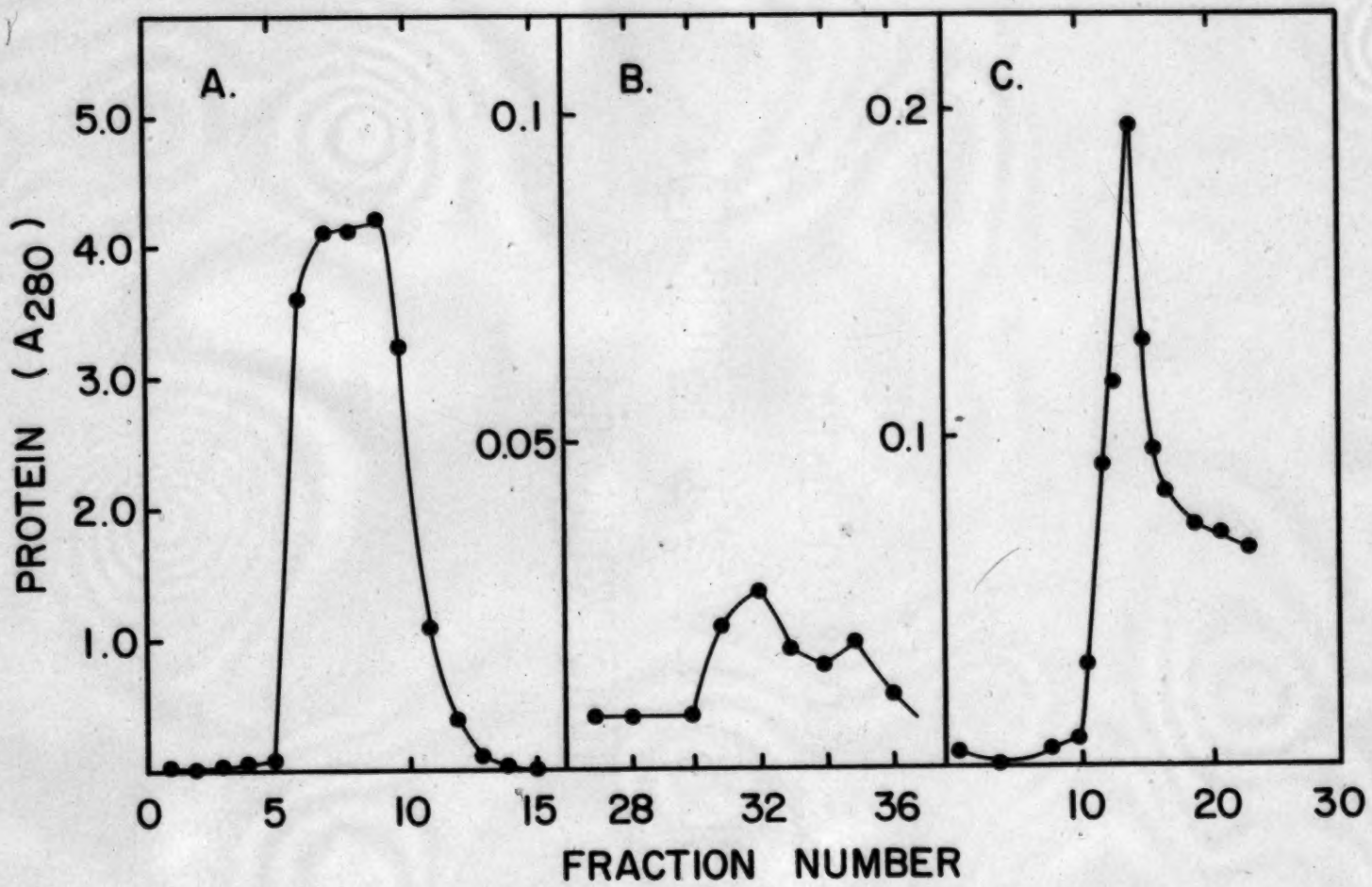

A.
B.
C.
PROTEIN (A280)
5.0
4.0
3.0
2.0
1.0
0.1
0.05
0.2
0.1
0
5
10
15
28
32
36
10
20
30
FRACTION NUMBER

Figure 16. Purification of Unactivated Precursor by Immunoabsorbance Chromatography Under Denaturing Conditions.

A. 7 ml of an $(NH_4)_2SO_4$ fraction of crude cell extract (32.2 units of precursor) were applied to a 28x4 cm immunoabsorbance column. Non-specific protein was eluted with 0.01 *M* Tris-HCl, pH 7.5, 5 ml fractions were collected, and A_{280} of each was measured.

B. Non-specific protein was further removed by elution with 5% formic acid, 5 ml fractions were collected, and the A_{280} was measured for each fraction.

C. Specifically bound protein was recovered by elution with 0.03 N NaOH and 2.5 ml fractions were collected into tubes containing 2.5 ml 10% formic acid. The A_{280} was measured for each fraction.

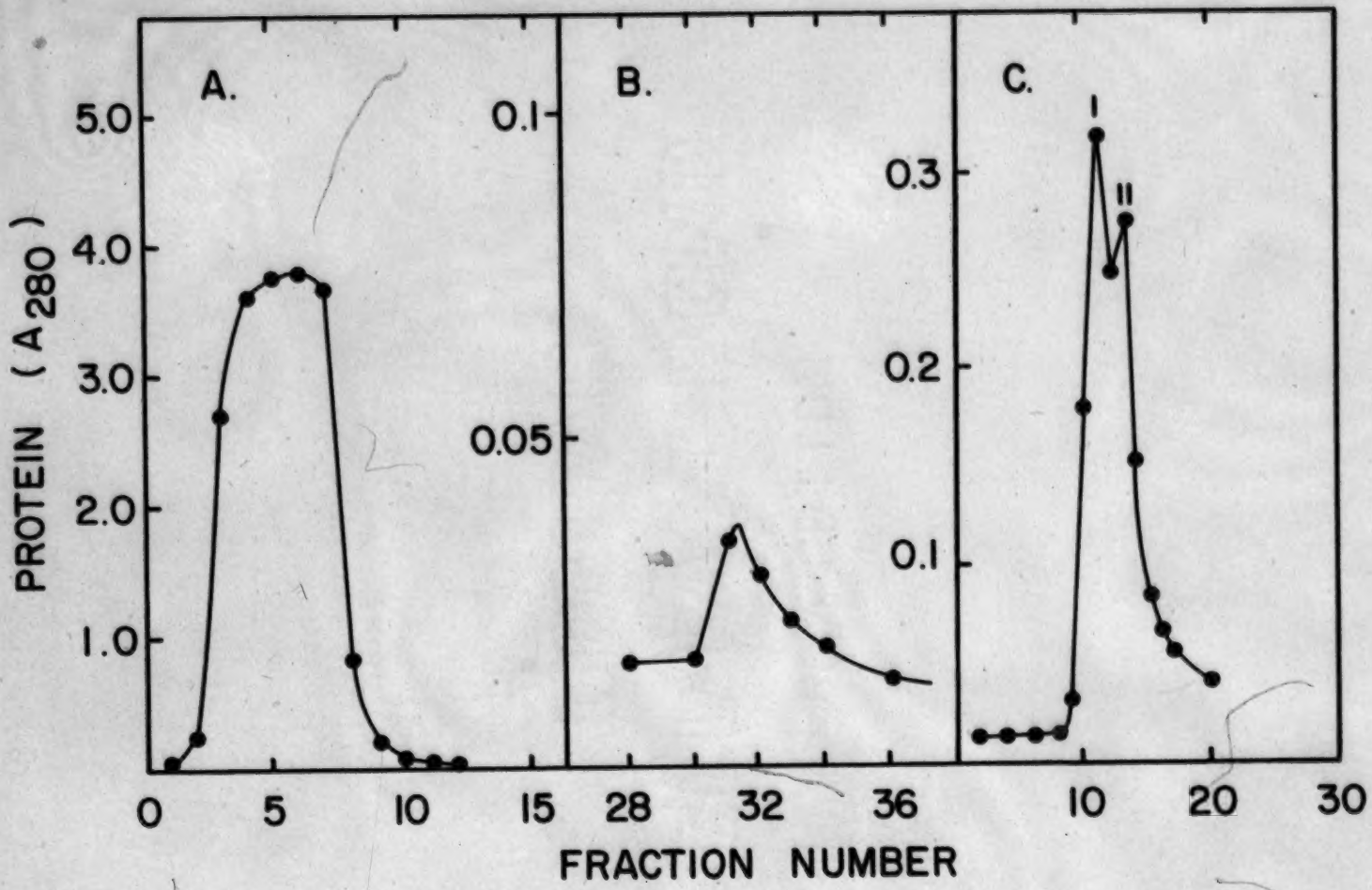

A.
B.
C.
PROTEIN (A_{280})
5.0
4.0
3.0
2.0
1.0
0.1
0.05
0.3
0.2
0.1
I
II
0
5
10
15
28
32
36
10
20
30
FRACTION NUMBER

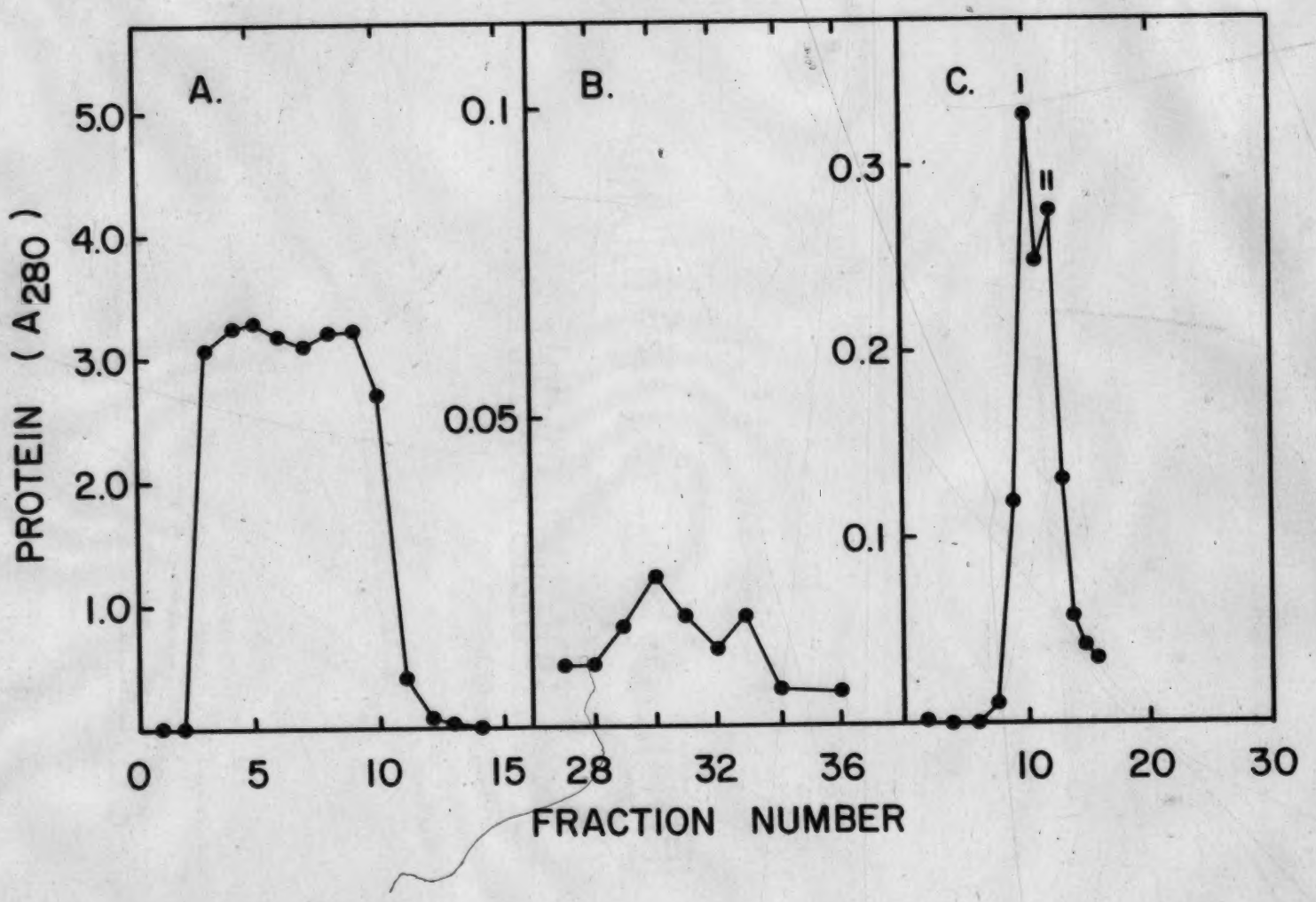
Figure 17. Purification of Protease 2 Activated Precursor by Immunoabsorbance Chromatography Under Denaturing Conditions.

A. 7 ml of an $(NH_4)_2SO_4$ fraction of crude cell extract (32.2 units precursor), previously activated with 0.403 mg protease 2, were applied to a 28x4 cm immunoabsorbance column. Non-specific protein was eluted with 0.01 *M* Tris-HCl, pH 7.5, 5 ml fractions were collected, and the A_{280} of each was measured.

B. Non-specific protein was further removed by elution with 5% formic acid, 5 ml fractions were collected, and the A_{280} was measured for each fraction.

C. Specifically bound protein was recovered by elution with 0.03 N NaOH and 2.5 ml fractions were collected into tubes containing 2.5 ml 10% formic acid. The A_{280} was measured for each fraction.

80a

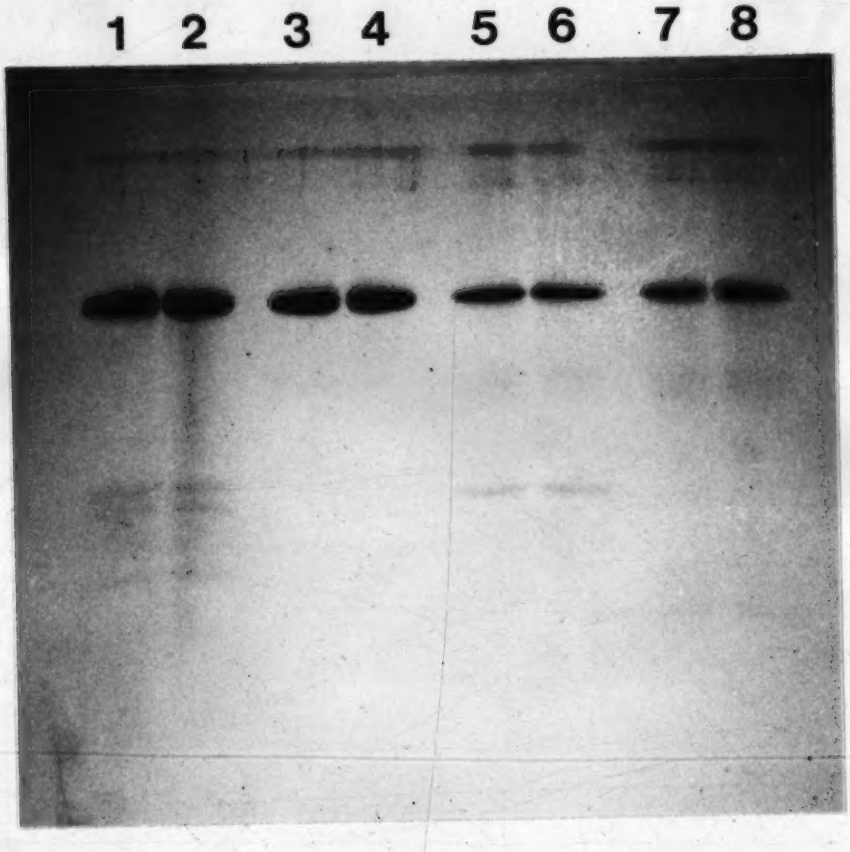

A.
B.
C.
I
II
PROTEIN (A280)
5.0
4.0
3.0
2.0
1.0
0.1
0.05
0.3
0.2
0.1
0
5
10
15
28
32
36
10
20
30
FRACTION NUMBER

were collected into tubes containing an equal volume of 10% formic acid (Panel C). Fig. 16C shows the elution profile of unactivated precursor. Two discernible peaks, I and II, resulted. Both peaks were collected and run on 10% polyacrylamide gels containing SDS to identify the precursor. Peak II was thus identified as the denatured precursor, now in virtually pure form (see below). Protease 2-activated precursor gave an identical elution profile to that of unactivated precursor when treated in the same manner (Fig. 17C). Again, peak II represented all of the activated precursor material. Protease 1 treated in the same way gave a single sharp peak corresponding to peak II in the previous experiment (Fig. 15C).

C. Size Characterization of Purified Proteins: The three preparations, purified under the denaturing conditions described above (*i.e*, equivalent to peak II), were analyzed by gel electrophoresis in 20% polyacrylamide in the presence of SDS. The results are shown in Plate VIII. Wells 1 and 2, which contained protease 1 purified by conventional means, show the major band of enzyme plus the faint bands representing autodigestion products. The three proteins purified by elution with formic acid (see above) gave rise to single bands. The fact that all three bands migrated identically indicates that there is no significant difference in molecular weight between the three proteins.

PLATE VIII 20% polyacrylamide gels in SDS of protease 1, unactivated precursor, and protease 2-activated precursor.

The above proteins were purified under denaturing conditions as described in Chapter II and run in duplicate on a 20% polyacrylamide gel in SDS. Tracks 1 & 2 contain protease 1, purified under non-denaturing conditions which is used as a reference protein, 3 & 4 contain protease 1, 5 & 6 contain unactivated precursor, and 7 & 8 contain protease 2-activated precursor.


1 2 3 4 5 6 7 8

The virtual absence of the autodigestion products seen in sample wells 3 and 4 attests to the efficacy of the denaturing procedure in preventing proteolysis during purification. To confirm this identity of the electrophoretic mobility of unactivated precursor and protease 1, peak II fractions of each were mixed and subjected to co-electrophoresis as previously described. No resolution of the two proteins was possible.

D. N-terminal Analyses: These pure denatured preparations of protease 1, unactivated precursor, and protease 2-activated precursor were further compared by identification of the N-terminal amino acid residue in each, as described in Chapter II. In each case, only α-dansyl alanine was detected.

III. IMPLICATIONS TO MECHANISM OF SECRETION

The fact that unactivated precursor, activated precursor and active enzyme do not differ from one another with respect to N-terminal residue or molecular weight clearly indicates that unactivated precursor does not contain an N-terminal signal peptide which is lost upon activation. These results do not exclude the possibility that an internal signal sequence may exist in the precursor to allow for its specific transport through the outer membrane. Such an internal sequence has been reported for chicken ovalbumin

[52]. Be that as it may, the foregoing results clearly show that activation of the precursor occurs without detectable reduction in size. This implies that activation may be a result of a change in the conformation of the precursor, causing its active site to be exposed during or following secretion. Experiments in support of such a mechanism are described in Chapter VI.

CHAPTER VI

MECHANISM OF ACTIVATION AND SECRETION OF THE PRECURSOR OF AN EXOCELLULAR PROTEASE OF *Pseudomonas aeruginosa* 34362A

I. INTRODUCTION

This part of the study is concerned with the mechanism of activation and secretion of the precursor through the outer membrane. Since little is presently understood about the transport of exocellular proteins in Gram-negative bacteria in general, the conclusions presented here may be of broader significance.

Experiments described in the preceding chapter showed that the precursor and its active counterpart have no significant difference in molecular weight, and that both contain N-terminal alanine, thereby eliminating proteolytic processing of the precursor as an integral part of the activation mechanism. Furthermore, these facts are not compatible with the involvement of an N-terminal prepiece that may be responsible for directing the secretion of the precursor before or during its activation.

This Chapter describes activation of the precursor by a diversity of mild conditions (including treatment with a variety of proteases and with certain detergents), as well as a measurement of differences in net charge and hydrophobicity of protease 1 and its precursor. The results lend strong support to a mechanism of activation involving a conformational change in the precursor. Further implications for secretion are discussed.

II. RESULTS

A. Activation of Precursor by Purification: As was shown in Chapter IV, the following procedures all led to activation of precursor: gel filtration, ion exchange chromatography, dialysis against neutral buffers and immune affinity chromatography. In the case of DEAE-cellulose chromatography, although precursor was active when assayed, it is significant that it is eluted earlier than protease 1 (see Fig. 5). Protease 1 has been shown to be eluted at a NaCl concentration of 0.08 *M* [12]. However, if the now-activated precursor (Peak 1) is rechromatographed, its behaviour is identical to that of protease 1 (Fig 18). Clearly, a change in the effective net charge of the precursor has accompanied its activation.

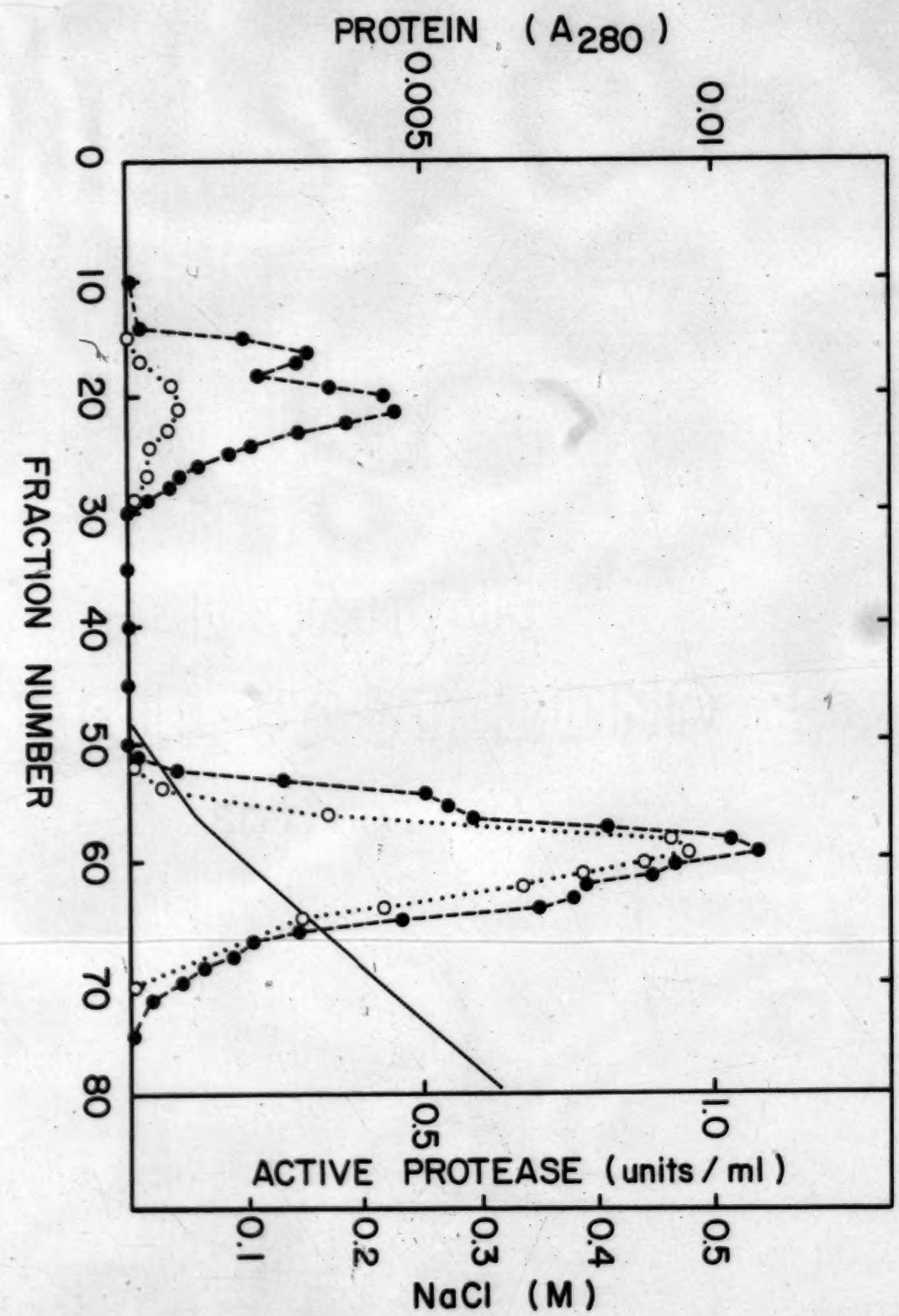
Figure 18. Rechromatography of Partially Purified Precursor (Peak I, Fig.5) on DEAE-cellulose.

Peak tubes of active precursor (protease) from the experiment of Fig. 5 were pooled and reapplied to a second DEAE-cellulose column as described in Chapter II. All fractions were assayed for protease activity, A_{280}, and NaCl concentration.

Protein (A_{280}): ●—–—●

Protease activity (units/ml): O••••O

NaCl (*M*): ——


PROTEIN (A_{280})
0.005
0.01
0
10
20
30
40
50
60
70
80
FRACTION NUMBER
0.5
1.0
ACTIVE PROTEASE (units / ml)
0.1
0.2
0.3
0.4
0.5
NaCl (M)

B. Activation of Precursor by Various Proteases:

(i) Specificity: The specificity of the proteolytic activation of precursor was further investigated by comparing the efficiency of activation by proteases of diverse specificities. The results are shown in Table VII. The most effective was protease 2, the second major exocellular protease produced by this organism. Active protease 1 is slightly less effective (autoactivation). The fact that all of these enzymes will catalyze activation, albeit with varying efficiencies, suggest that proteolytic activation is not restricted to cleavage of a specific peptide bond.

(ii) Kinetics of proteolytic activation: The kinetics of activation of crude unactivated precursor by protease 2 were studied by exposure of a small amount of unactivated precursor (0.02 units) to 50 ug of protease 2 and measuring the amount of active enzyme released at time intervals. The results are shown in Fig. 19, curve 1. A slight lag period of about 1 min is observed before a sharp increase in the rate of conversion of precursor to active protease is seen.

When the amount of protease 2 present is reduced from 50 to 25 ug (see Fig. 19, curve 2), two observations can be made. Firstly, the lag period is increased two-fold, and secondly, there is a decrease in the maximum rate of

Table VII

Activation of Precursor by Various Proteolytic Enzymes

Enzyme	Amount (mg) required to activate 1 unit of precursor in 15 minutes
Protease 2	0.011
Protease 1	0.071
Subtilisin Type VII	0.094
Pronase	0.134
Trypsin	0.149
Chymotrypsin	0.349

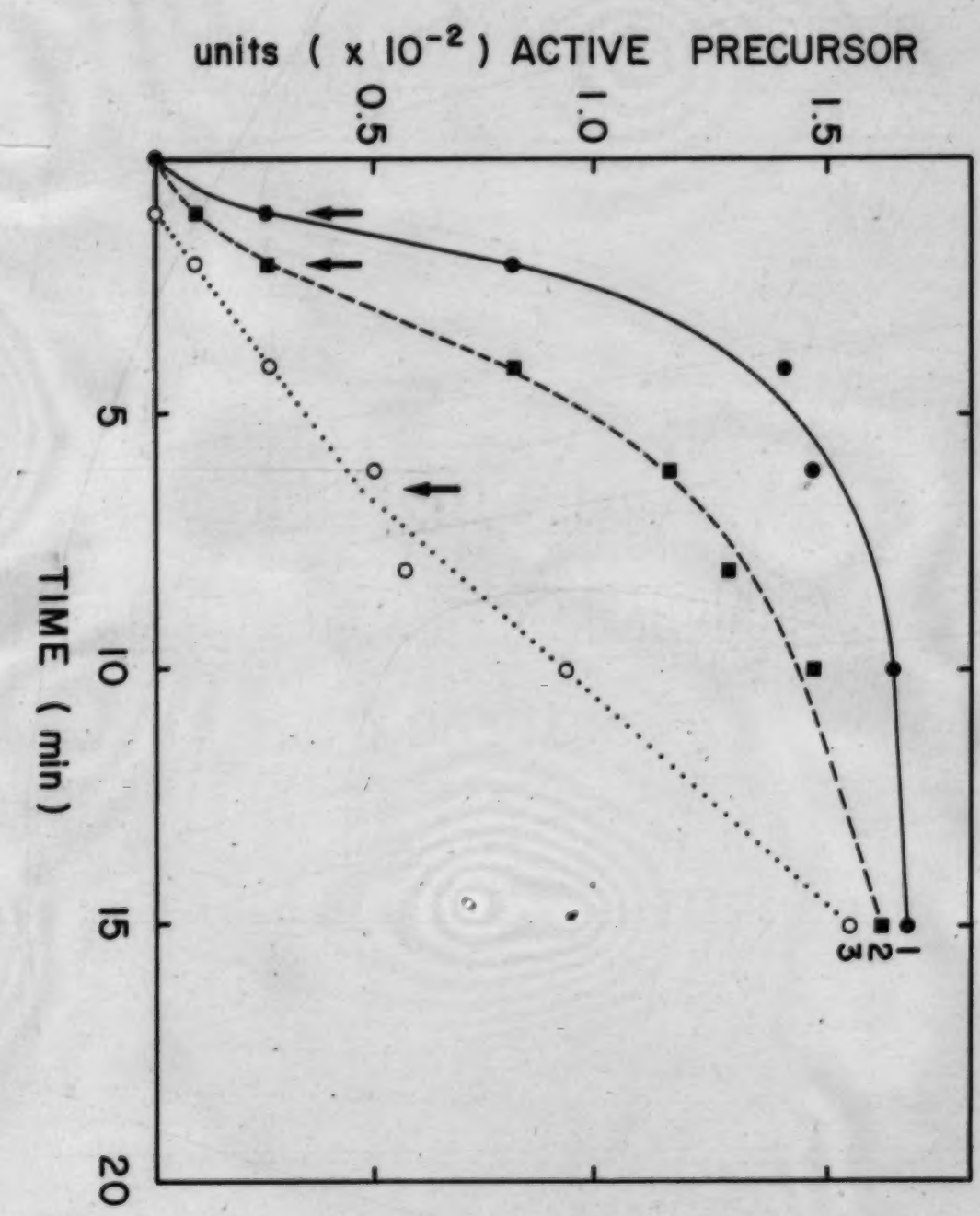
Figure 19. Kinetics of Activation of Precursor by Protease 2.

1. 2 mg of crude cell extract was added to 1 ml of 0.02 *M* Tris-HCl, 2 m*M* $CaCl_2$, pH 7.5 and prewarmed to 37°C. To this was added 0.045 mg of protease 2 and the resulting mixture was incubated for the times indicated. After each time interval, the mixture was assayed for new proteolytic activity using azocasein as the substrate (see Chapter II).

2. The above protocol was followed except that only 0.022 mg of protease 2 was added.

3. The above protocol was followed except that 4 mg of crude cell extract and 0.022 mg of protease 2 was used.

The arrows indicate the end of the lag period and the onset of the maximum rate of activation.


units (x 10⁻²) ACTIVE PRECURSOR
0.5
1.0
1.5
TIME (min)
5
10
15
20
1
2
3

activation from 0.54 to 0.28 units/min for curves 1 and 2, respectively.

When the precursor:protease 2 ratio was altered by doubling the precursor concentration, there was an unexpected three-fold increase in the lag period, coupled with a decrease in the maximum rate from the expected 0.28 to 0.13 units/min (Fig. 19, curve 3). The pronounced lag, clearly evident under these conditions, suggests the possibility of a two-stage reaction, the first step involving the essential removal of an inhibitory molecule found in the crude preparation, followed by the activation step *per se*. For example:

CRUDE UNACTIVATED PRECURSOR

↓ proteolytic removal of stabilizing protein(s)

"MODIFIED" PRECURSOR

↓ spontaneous activation

ACTIVE PROTEASE 1

C. Non-proteolytic Activation of Precursor:

(i) Specificity: Since it was apparent that activation is more complex than a simple proteolytic cleavage of an N-terminal signal peptide, it was decided to

try controlled alteration of molecular configuration as a factor in activation. To this end, crude unactivated precursor was treated with the reducing and denaturing agents listed in Table VIII. It was found that reducing agents (dithiothreitol and 2-mercaptoethanol) and certain denaturing agents (urea, Triton-X100, Brij 35, Tween 80 and cholate) were ineffective in activating precursor. The combination of a denaturant (urea) and a reducing agent (dithiothreitol) which was reported to activate *Pseudomonas* exotoxin, another exocellular protein [8], was ineffective in this case, even at elevated concentrations. However, the three anionic detergents, N-lauroyl sarcosine, sodium dodecyl sulfate and deoxycholate, were very effective, resulting in at least 90% activation of precursor. 2 *M* guanidine-HCl was somewhat less effective (45% activation) but it is likely that some complete denaturation has occurred since the use of higher concentrations resulted in total destruction of precursor rather than augmented activation.

These results show that activation of precursor occurs under certain mild denaturing conditions that would be expected to produce a change in protein conformation less extensive than complete denaturation.

Table VIII

Activation of Precursor by Various
Denaturing and Reducing Agents

Reagent	% Activation
1. Denaturing Agents	
1 M urea (2M)	0
1% Triton X-100	0
0.5% Brij 35	0
0.5% Tween 80	0
0.5% cholate	0
2 M guanidine-HCl	45
0.5% N-lauroyl sarcosine	92
0.5% sodium dodecyl sulfate	95
0.5% deoxycholate	95
2. Reducing Agents	
5 mM dithiothreitol (25 mM)	0
3. Denaturing & Reducing Agents	
1 M urea + 12.5 mM dithiothreitol	0

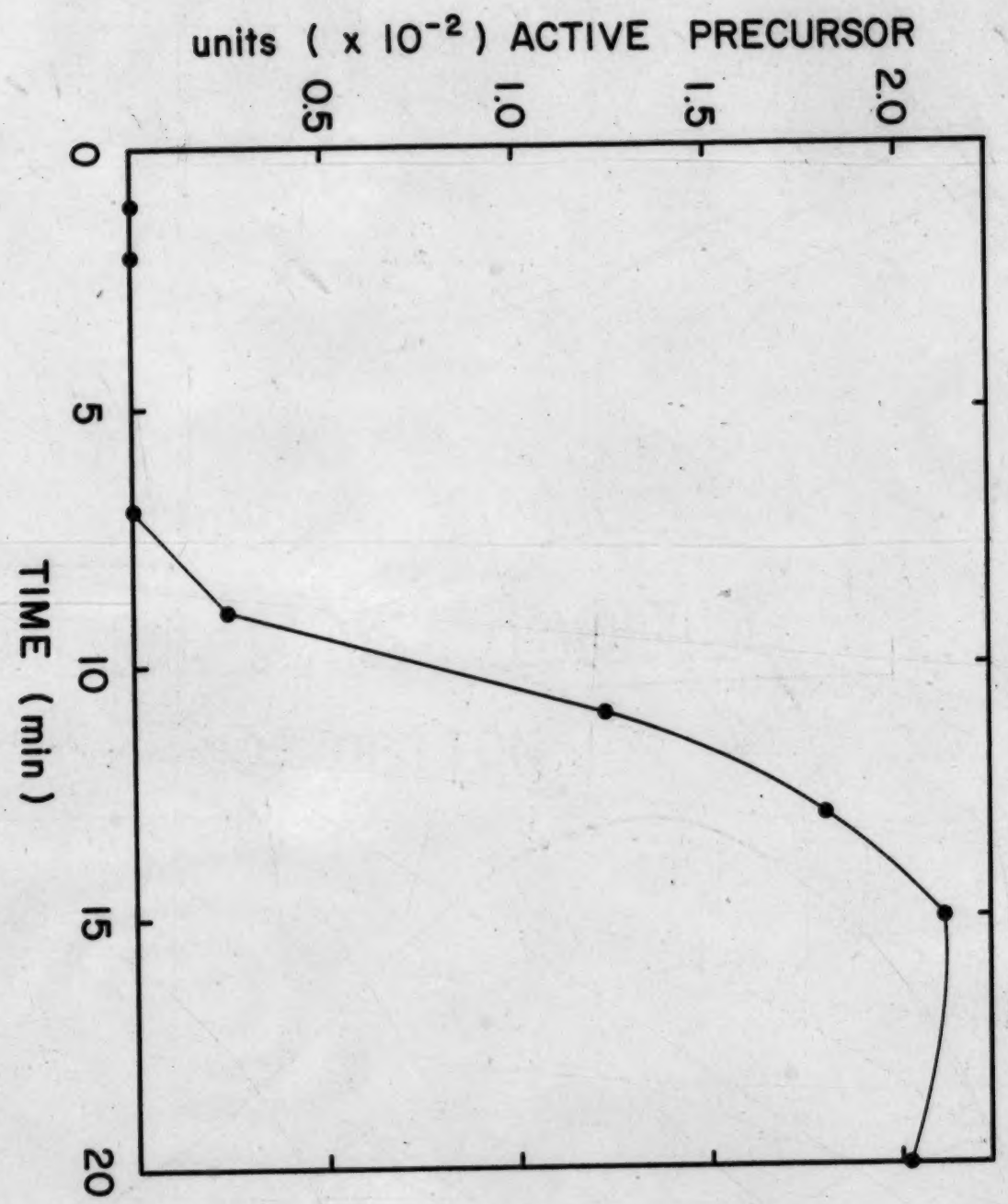
(ii) Kinetics of non-proteolytic activation of precursor: For this study, the anionic detergent N-lauroyl sarcosine in the precursor:detergent ratio which gave maximum activation (95%) was chosen and the degree of activation was measured at timed intervals. The results in Fig. 20 show a long lag of 7 min before activation begins. This reveals a process more complex than a simple non-cooperative 1:1 combination of precursor and detergent. Undoubtedly, once activation begins, a cascade effect results from the newly-activated precursor contributing to activation of other precursor molecules. The result is virtually complete activation within a 6 min period.

D. Evidence for a Conformational Change of Precursor upon Activation: If a conformational change occurs that results in the unmasking of the active site of the protein, this transition might be accompanied by exposure or burial of hydrophobic or polar regions of the molecule with a resulting change in the hydrophobicity of the enzyme upon activation.

Conventional conformational studies (using circular dichroism, for example) require highly purified samples. Since purification of the precursor without activation is not possible, another approach was tried. In this experiment, unactivated precursor and protease 2 activated

Figure 20. Kinetics of Activation of Precursor by N-Lauroyl Sarcosine.

A mixture of 0.5 ml of cell extract (16.4 mg) and 0.5 ml of 1% N-lauroyl sarcosine was incubated at 37°C for the times indicated. After each time interval, 10 µl of the mixture was assayed for new proteolytic activity using azocasein as the substrate (see Chapter II).


units (x 10^{-2}) ACTIVE PRECURSOR
0.5
1.0
1.5
2.0
0
5
10
15
20
TIME (min)

precursor were chromatographed separately on octyl-Sepharose columns (see Chapter II). Fig. 21 shows the elution profile for unactivated precursor. It can be seen that precursor was bound strongly to the octyl-Sepharose and was eluted at 0.76 *M* $(NH_4)_2SO_4$ (tube 60-70), now in active form. In contrast, protease 2 activated precursor bound to the octyl-Sepharose less tenaciously (Fig. 22) as evidenced by its elution from the column at 0.87 *M* $(NH_4)_2SO_4$ (tube 50-60). As expected, other proteins in the protease 2 activated precursor preparation eluted differently in the unactivated precursor preparation. This undoubtedly results from proteolytic modification of the hydrophobic properties of some of these contaminants. This implies that unactivated precursor and exocellular protease 1 differ significantly in structure as reflected by the change in hydrophobicity that accompanies activation. Together with the differences in charge detected by DEAE-cellulose chromatography (see above), these data support the concept that the precursor, upon activation, adopts the structural characteristic of protease 1 and that a conformational change is an essential part of the activation mechanism.

Figure 21. Elution Profile of Unactivated Precursor Chromatographed on Octyl-Sepharose.

3 ml (27 units of precursor) of an $(NH_4)_2SO_4$ fraction of crude cell extract were applied to an octyl Sepharose column as described in Chapter II. 5 ml fractions were collected and assayed for precursor and protease activity, UV absorbance, and $(NH_4)_2SO_4$ concentration.

Protein (A_{280}): ■—■

Precursor activity (units/ml): ●--●

Ammonium sulfate concentration (*M*): •••••

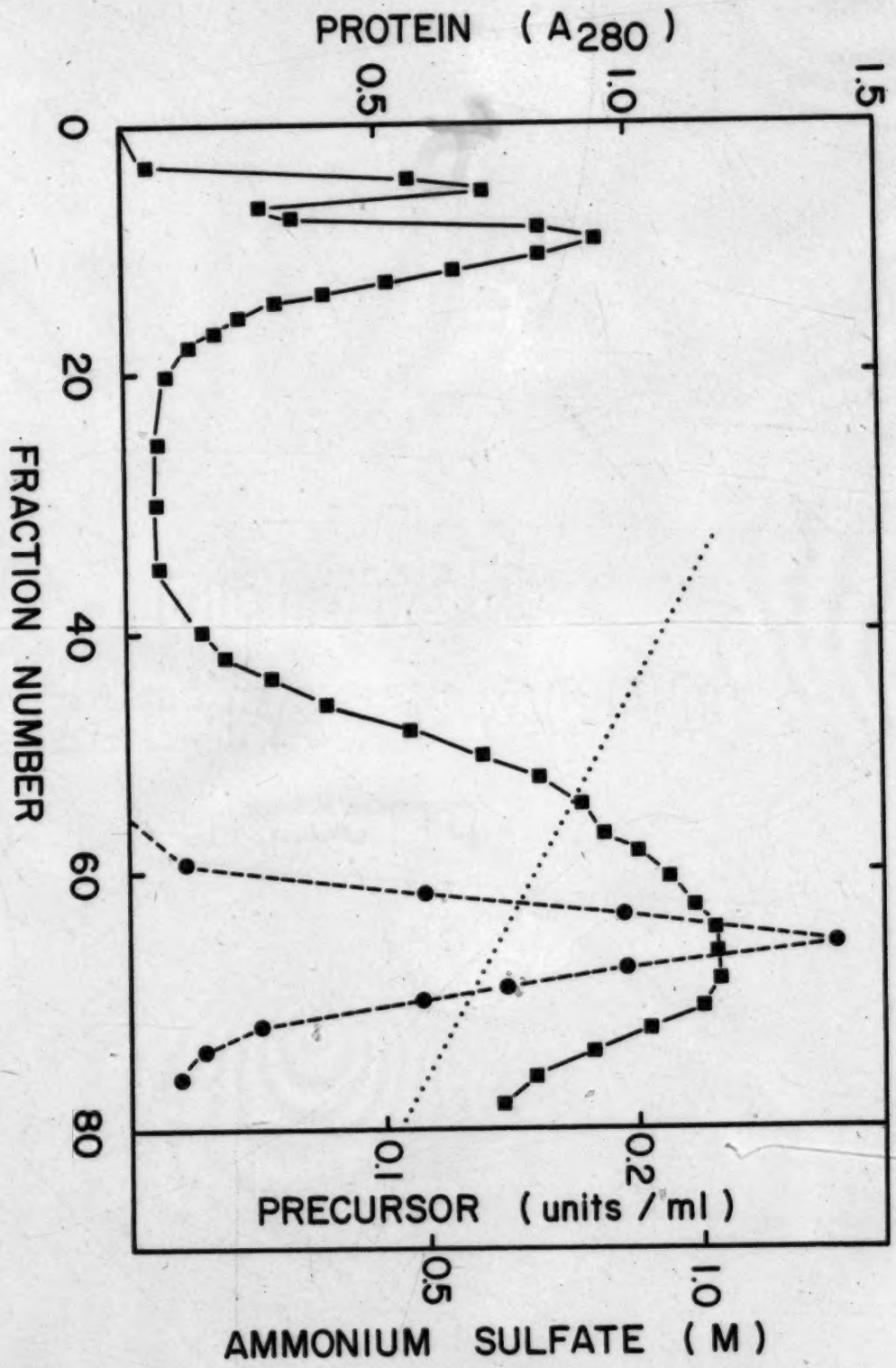

PROTEIN (A280)
0.5
1.0
1.5
0
20
40
60
80
FRACTION NUMBER
0.1
0.2
PRECURSOR (units / ml)
0.5
1.0
AMMONIUM SULFATE (M)

Figure 22. Elution Profile of Protease 2 Activated Precursor Chromatographed on Octyl-Sepharose.

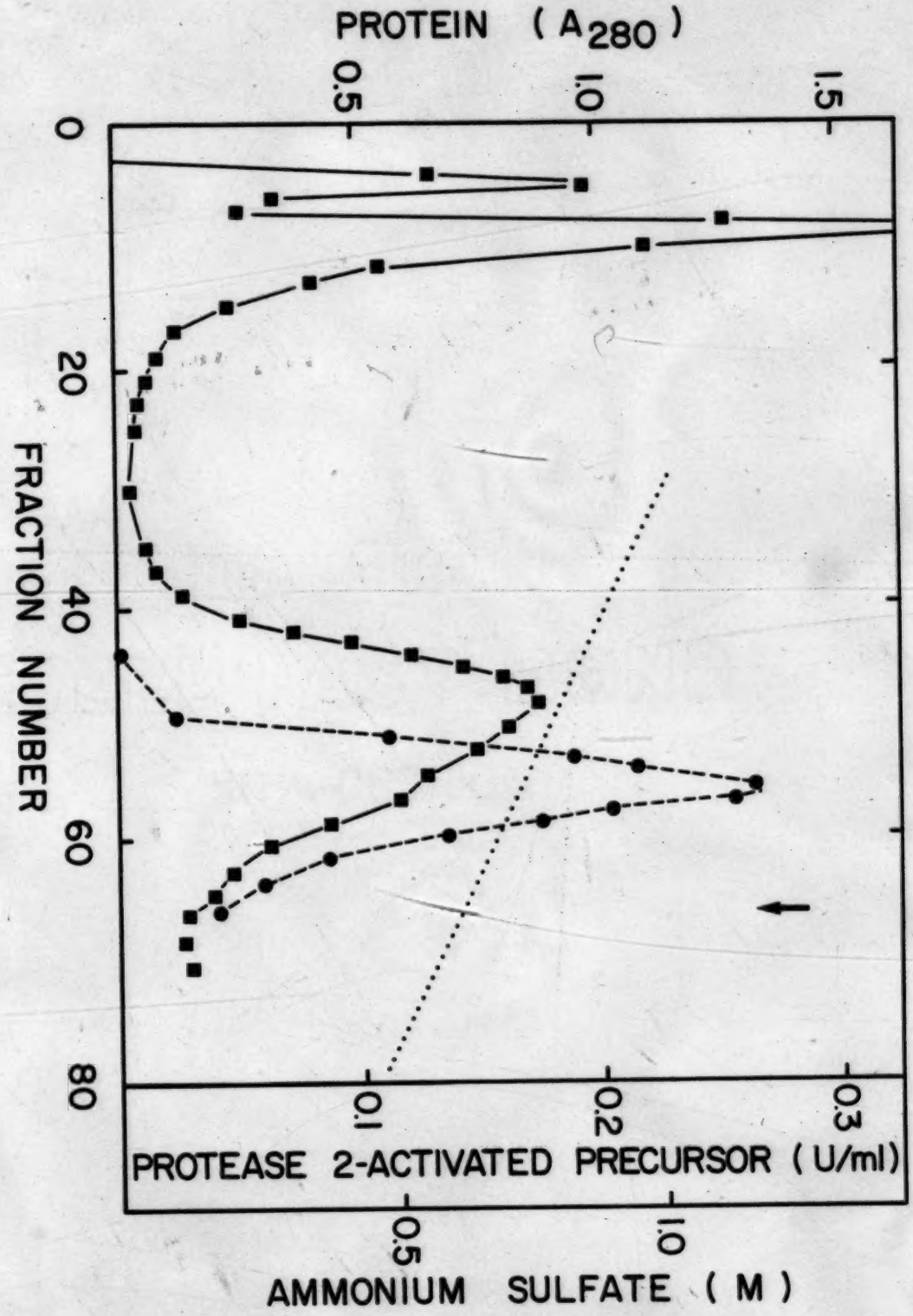
3 ml (27 units of precursor) of an $(NH_4)_2SO_4$ fraction of crude cell extract, previously activated with 0.182 mg of protease 2, were applied to an octyl Sepharose column as described in Chapter II. 5 ml fractions were collected and assayed for precursor and protease activity, UV absorbance, and $(NH_4)_2SO_4$ concentration.

Protein (A_{280}): ■—■

Protease 2 activated precursor activity (units/ml): ●--●

Ammonium sulfate concentration (*M*): .....

Arrow: unactivated precursor


PROTEIN (A280)
0.5
1.0
1.5
0
20
40
60
80
FRACTION NUMBER
0.1
0.2
0.3
PROTEASE 2-ACTIVATED PRECURSOR (U/ml)
0.5
1.0
AMMONIUM SULFATE (M)

III. DISCUSSION

The fact that activation of the precursor is not accompanied by a release of an N-terminal signal peptide, plus the fact that activation can be effected by a diversity of conditions, such as treatment of the crude preparation with a variety of proteases or with anionic detergents, by dialysis against neutral buffers, or by chromatographic separation on DEAE-cellulose, Sephadex G-100 and octyl-Sepharose, provide compelling evidence that a structural transition in the precursor is an integral part of the activation process.

Although the nature of this transition is not defined clearly, it is possible to interpret the total of these results in terms of a model for activation and secretion. Proteolytic nicking of the precursor itself, as is the case with the pancreatic zymogens, does not appear to be a requirement, since activation is not accompanied by a detectable change in molecular weight or N-terminus. The data do not eliminate the possibility that proteolytic activation involves removal of a very short region from the C-terminus of the precursor, but this possibility is considered to be an unlikely component of the activation process, especially in view of the other non-proteolytic conditions that can effect activation. More probably, the

activation by proteases is the consequence of change in the environment around the precursor. For example, if non-covalently associated surrounding protein(s) or peptide(s) were responsible for maintaining the inactive configuration of the precursor, their degradation by proteases would result in the precursor changing its conformation, thus exposing its active site. This is consistent with the observation that chromatographic separation of the precursor from other proteins results in activation. The kinetics of activation by both protease 2 and N-lauroyl sarcosine suggest that the process is complex, consisting of multiple steps. It is proposed that the activation step can proceed only after a preliminary "silent" reaction, such as the removal of aforementioned associated protein or peptide. The stability of precursor in crude preparations or upon dialysis in low pH or high ionic strength buffers, but its rapid activation by dialysis against neutral buffers, gives further support to the concept of the necessity for removal or disruption of a non-covalently associated species. Such removal alone is insufficient for activation *per se*; it is proposed that it permits the actual activation step *i.e.* a conformational change in the precursor. The evidence for this is that mild denaturation without proteolysis will cause activation, and more directly, the observations that the adsorption behaviour of the precursor on DEAE-cellulose

and on octyl-Sepharose alters upon activation, indicating a change in the effective surface charge and of the hydrophobicity, respectively, of the precursor concomitant with activation.

The demonstrated loss of hydrophobicity of the precursor upon activation permits some speculation about the interaction of the precursor with the hydrophobic components of the outer membrane. The fact that precursor binds so tightly to the hydrophobic octyl-Sepharose column suggests that it is in a suitable conformation for interaction with lipid components of the membrane. This may be of significance to the process of transport of the molecule through the outer membrane. It is an attractive possibility that the unactivated precursor may present an internal "signal" region to the membrane, as has been reported for ovalbumin secretion [62]. Furthermore, since precursor becomes activated upon elution from the column, the *in vivo* analogy may be extended one step further to say that the precursor is activated by the process of dissociation from the outer membrane component after transport. Transport of the precursor naturally interrupts its association with the stabilizing protein, causing a conformational change resulting in reduction of the hydrophobic interactions with the membrane and exposure of the active site. The net result is the release of active enzyme (protease 1) into the

exocellular milieu.

CHAPTER VII

PRELIMINARY GENETIC STUDIES ON PROTEASE 1-NEGATIVE MUTANTS

Several protease 1-negative mutants were examined for the presence of cell-associated precursor in the hope of finding a mutant that produced periplasmic precursor but was incapable of its secretion across the outer membrane. These mutants were of the wild-type strain, PAKS 1 of *P. aeruginosa*. PAKS 1 was shown to be similar to 34362A with respect to protease 1/precursor production (Table IX). Of those mutants examined, the majority were both protease 1-negative and precursor-negative (S.E. Jensen, personal communication). However, one mutant, PAKS 18, although producing little or no protease 1, was shown to yield large amounts of precursor. Therefore, a comparative study was carried out on PAKS 1, PAKS 18 and 34362A in order to determine the location of the precursor in PAKS 18. These results are presented in Table IX. The majority of the precursor present in PAKS 18 was found to be located in the periplasm outside the cytoplasmic membrane (82%). This is similar to the percentage of precursor found in the periplasm of PAKS 1 and 34362A. However, precursor in the wild-type strains normally represents only 10% of the total protease 1 at 12 hr. In PAKS 18, 85% of its protease 1 is in

Table IX

Localization of Precursor in Wild Type vs Mutant Cells

	Strain of Pseudomonas aeruginosa		
	34362A	PAKS 1	PAKS 18
1. Growth (A_{600})	8.2	7.34	6.6
2. Total extracellular protease 1 (units)	228	219	20
3. Total cell-associated precursor (units)	25.9	28.3	117
4. % precursor in periplasmic space	97	95	82
5. % precursor in periplasmic space vs total units (2 & 3)	10	11	85

the form of cell-associated precursor. Furthermore, at least four times as much precursor is present in PAKS 18 at 12 hours than in either 34362A or PAKS 1.

It thus appears that the protease 1 gene is being expressed in the PAKS 18 mutant but that the synthesized protease 1 precursor is not secreted from the periplasm to the culture supernatant. Two possible hypotheses can explain this phenomenon. Firstly, the mutation could be one involving the proteins in the outer membrane. Perhaps the secretion of precursor through the outer membrane requires a specific binding/transport protein; if this protein is missing, minimal secretion might be possible. Secondly, the mutation could be in the protease 1 gene itself. Although the gene is still being expressed, the protein synthesized may be sufficiently altered so that it will not be in the correct conformation in the periplasm to allow its proper interaction with the outer membrane. Therefore, secretion would not occur.

Further investigation is required to determine the nature of the genetic lesion in PAKS 18. Analyses of the outer membrane proteins of PAKS 1 and PAKS 18 may provide some insight into the possibility of a transport protein being involved in the secretion mechanism. Moreover, the amino acid composition of the precursor found in PAKS 18 may

provide interesting information regarding the conformation of the precursor as compared to the precursor of wild-type cells. Clearly, further studies of these mutants may be a fruitful approach to provide details about the activation and secretion of precursor.

CHAPTER VIII

GENERAL CONCLUSIONS

The foregoing results, although not sufficient to allow for complete description of a mechanism of secretion of active protease 1 from *P. aeruginosa*, do permit several important conclusions to be made that place constraints on our view of this process.

Clearly, the results are not easily compatible with the involvement of an N-terminal signal sequence in the transport of the protease 1 precursor through the outer membrane. This of course does not preclude the possibility that at some stage of protease 1 synthesis a prepiece is present. A recent paper by Beckwith *et al*. [83] on the secretion of periplasmic and outer membrane proteins of *E. coli* demonstrated that most of these proteins do require a prepiece for transport across the cytoplasmic membrane, and their data also suggested that these proteins share a common step in localization on the cytoplasmic membrane before the polypeptide becomes accessible to the processing enzyme. Protease 1 may be an exception (as is the case for ovalbumin [52]) but more likely it is also initially synthesized with an N-terminal signal region. In order to

determine this, the use of cell-free synthesis of protease 1 followed by immunoprecipitation would probably be the best route to take. These types of studies could yield very exciting information that could provide a complete picture of protease 1 secretion.

Nevertheless, the question of how protease 1 is secreted across the outer membrane is partially answered in this thesis. The precursor form of protease 1 isolated in the periplasmic space appears to differ from its active counterpart by virtue of its conformation. It appears that precursor is a more compact molecule which is less charged and more hydrophobic than protease 1. This conformation may be necessary in aiding recognition between the protein and a specific site on the outer membrane or for allowing the molecule to interact directly with the lipid bilayer. The fact that the precursor, which is normally found in small amounts in the periplasm (*i.e.*, 10% of the total protease 1 at 12 hours of growth), greatly accumulates in the periplasmic space in the protease 1-negative mutant PAKS 18 reduces the possibility that protease 1 is being secreted through so-called "Bayer's Junctions" [84] (*i.e.*, proposed fusion points between the inner and outer membrane).

Figure 23 illustrates a mechanism of secretion of the protease 1 precursor across the outer membrane that is

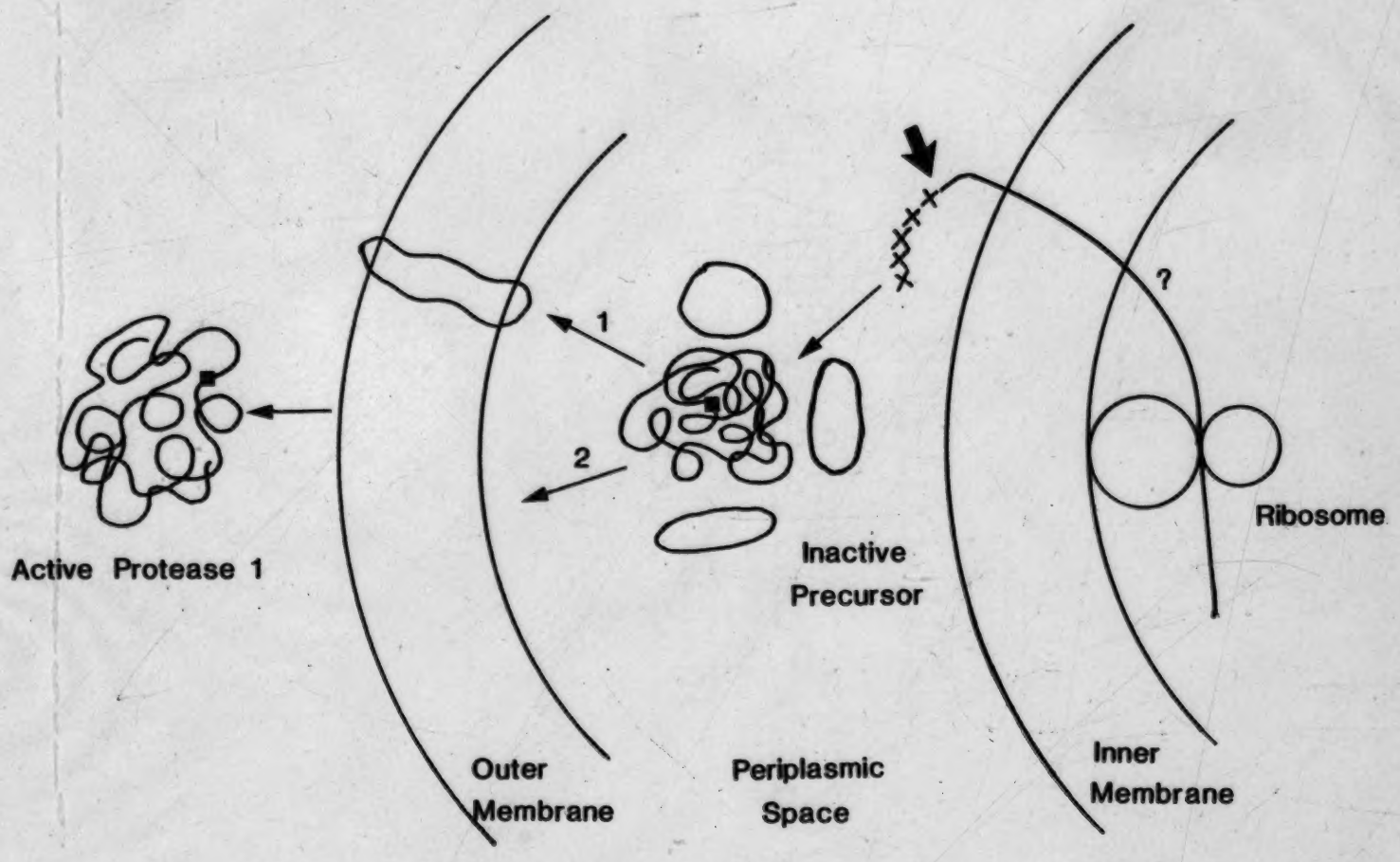
Figure 23. Proposed Mechanism for the Secretion of Protease 1 Precursor Through the Outer Membrane.

This scheme shows protease 1 being initially synthesized on membrane-bound ribosomes with an N-terminal leader sequence (XXXXX). Although this is not known for certain, it appears that this is the most likely mechanism for its secretion through the inner (cytoplasmic) membrane in light of the literature to date. Secretion through the inner membrane is presumed to be followed by proteolytic processing (➡) since it has been shown that the protease 1 precursor in the periplasmic space does not contain a prepiece.

The precursor in the periplasm is depicted as being in a more compact conformation whereby its active site (■) is buried. Surrounding proteins help keep the precursor in this inactive conformation. Secretion across the outer membrane may occur by two possible mechanisms: 1. there may exist in the outer membrane a specific transport protein or 2. the precursor may interact directly with the lipid bilayer.

Once the precursor has been secreted through the outer membrane, it assumes its native conformation (protease 1) whereby its active site will now be exposed.

109a


1
2
?
Active Protease 1
Inactive
Precursor
Ribosome
Outer
Membrane
Periplasmic
Space
Inner
Membrane

compatible with our current knowledge of this process (see legend for explanation). It is clear that this work and parallel studies described in the recent literature point to the need for further elucidation of other factors which may play a role in deciding the location of proteins in Gram-negative bacteria once they have traversed the cytoplasmic membrane.

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APPENDIX I

During the course of this work, I also contributed to the completion of a published study on nutritional factors controlling exocellular protease production by *Pseudomonas aeruginosa* [S.E. Jensen, I.T. Fecycz and J.N. Campbell (1980) *J. Bacteriol.*, 144:844-847]. My contribution principally involved the development of a modified technique for localization proteolytic activity on polyacrylamide gels.